Nicotinamide Inhibits IRF-1 mRNA Induction and Prevents IL-1 β-induced Nitric Oxide Synthase Expression in Pancreatic β cells

博 士 論 文

Nicotinamide Inhibits IRF-1 mRNA Induction and Prevents IL-1 β-induced Nitric Oxide Synthase Expression in Pancreatic β cells.

（ニコチン酸アミドは膵β細胞においてIRF-1 mRNAの誘導を阻害し、IL-1 β誘起性NO合成酵素の発現を抑制する）

東北大学大学院医学研究科 外科学系専攻
脳神経外科

赤 羽 敦 也

(1)

Nicotinamide Inhibits IRF-1 mRNA Induction and Prevents IL-1β-induced Nitric Oxide Synthase Expression in Pancreatic β cells

Atsuya Akabane

Department of Neurosurgery, Tohoku University School of Medicine,

Sendai, Japan

SUMMARY

Nitric oxide produced by inducible nitric oxide synthase in islets exerts inhibitory and cytotoxic effects on pancreatic β cells and is therefore thought to be a potent mediator in the pathogenesis of insulin-dependent diabetes mellitus. Here, using isolated rat pancreatic islets, I showed that high-concentration nicotinamide (20 mM) but not low-concentration nicotinamide (5 mM) attenuated the interleukin-1β-evoked inhibition of glucose-induced insulin secretion by preventing the induction of interferon regulatory factor-1, a transcriptional factor which plays an essential role in inducible nitric oxide synthase gene expression, and the interleukin-1β-induced nitric oxide formation. High-concentration nicotinamide also restored an interleukin-1β-induced decrease in ATP content in pancreatic β cells, suggesting that interleukin-1β-induced nitric oxide inhibits the mitochondrial function. The present results show the molecular basis of the preventive effect of high-dose nicotinamide on insulin-dependent diabetes mellitus.

Abbreviations: PARS, poly(ADP-ribose) synthetase; 3AB, 3-aminobenzamide; IDDM, insulin-dependent diabetes mellitus; IL-1β, interleukin-1β; NO, nitric oxide; NMMA, N^G-monomethyl-L-arginine; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; NFκB, nuclear factor κB; IRF-1, interferon regulatory factor-1; 1,5-DHIQ, 1,5-dihydroxyisoquinoline; FCS, fetal calf serum; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

INTRODUCTION

Okamoto et al. have previously found that streptozotocin and alloxan, which produce diabetes mellitus in experimental animals, cause DNA strand breaks which activate nuclear poly(ADP-ribose) synthetase (PARS) (Yamamoto et al. 1981; Uchigata et al. 1982; Okamoto 1990). The activation of PARS depletes intracellular NAD^+ and inhibits pancreatic β cell functions such as insulin synthesis, and β cell ultimately dies. PARS inhibitors such as nicotinamide and 3-aminobenzamide (3AB) reverse the reduction of the NAD^+ level and also the inhibition of insulin biosynthesis (Yamamoto et al. 1981; Uchigata et al. 1982; Okamoto 1990). Recent studies using PARS knock-out mice confirmed that PARS activation is a major cause of NAD^+ depletion and subsequent β cell death (Heller et al. 1995).

Many lines of evidence indicate that insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease (Mandrup-Poulsen et al. 1990; Cooke 1990). Early stage of IDDM is characterized by infiltration of lymphocytic cells (Gepts 1965), followed by an inhibition of insulin secretion and ultimately selective destruction of β cells of the pancreatic islet (Mandrup-Poulsen et al. 1985, 1986). Macrophage infiltration has been observed early stage in the disease process, and activated macrophages secrete various cytokines including interleukin-1β (IL-1β). IL-1β has been reported to inhibit islet cell functions such as glucose-induced insulin secretion by increasing nitric oxide (NO) formation *in vitro* (Sandler et al. 1987; Corbett et al. 1991). N^G-monomethyl-L-arginine (NMMA), NO synthase (NOS) inhibitor, prevents both IL-1β-induced NO formation and inhibition of insulin secretion (Corbett et al. 1991). Therefore, IL-1β and NO formation are proposed to be important mediators of pancreatic β cell damage in IDDM.

NO, a short-lived reactive radical, has recently been found to mediate a large number of diverse physiologic functions in many organs (Moncada et al. 1991). In the immune system, NO is released from activated macrophages and contributes to the antimicrobial and tumoricidal activity of the macrophages (Nathan and Hibbs 1991;

Nathan 1992). However, excessive NO production in various tissues can contribute to functional disturbance and ultimately cell death (Moncada 1992).

The generation of NO from L-arginine is catalyzed by NOS. Molecular cloning and sequencing analyses revealed the existence of at least three main types of NOS isoforms. A neuronal NOS, a 160 kDa-protein, displays recognition sites for the three redox cofactors NADPH, FAD, and FMN (Bredt et al. 1991). A cDNA encoding endothelial NOS, a 133-kDa protein, was cloned from endothelial cells (Janssens et al. 1992). The neuronal and endothelial NOS are 60% identical at the amino acid level. These isoforms are constitutively expressed and their activity is activated by Ca^{2+} and calmodulin. The third type, inducible NOS (iNOS), has been cloned from vascular smooth muscle cells (Nunokawa et al. 1993) and its activity is Ca^{2+}- and calmodulin-independent. The iNOS mRNA is highly induced in macrophages, hepatocytes and pancreatic β cells by bacterial endotoxin and cytokines such as IL-1β.

IL-1β, produced by activated macrophages *etc.*, induces iNOS in pancreatic islets (Green et al. 1994). NO, thus produced by iNOS in islets, exerts inhibitory and cytotoxic effects on pancreatic β cells (Green et al. 1994). Recent studies have shown that NO produced in large amounts by iNOS causes DNA strand breaks (Delaney et al. 1993) and NAD^+ depletion (Bolaffi et al. 1994), leading to islet cell death (Sandler et al. 1987; Ankarcrona et al. 1994). Low-concentration of nicotinamide (5 mM), which almost completely inhibits PARS activity (Yamamoto and Okamoto 1980), prevents the IL-1β-induced NAD^+ depletion and islet cell death (Andersen et al. 1994). However, it fails to prevent the IL-1β-induced inhibition of glucose-induced insulin secretion (Andersen et al. 1994). In contrast, high-concentration nicotinamide (20 mM) attenuates the IL-1β-induced inhibition of glucose-induced insulin secretion (Andersen et al. 1994).

The promoter sequence of the murine iNOS gene (Xie et al. 1993) contains cytokine-responsive elements for the binding of transcriptional factors such as interferon regulatory factor-1 (IRF-1) (Miyamoto et al. 1988). The recent study using IRF-1 knock-out mice indicated that IRF-1 is absolutely essential for iNOS mRNA induction

(Kamijo et al. 1994). In the present study, I showed that high-concentration nicotinamide inhibited the induction of IRF-1, a transcriptional factor which is essential for iNOS gene expression (Kamijo et al. 1994), thereby attenuating the NO-induced β-cell dysfunction.

MATERIALS AND METHODS

Islets Isolation and Culture – Pancreatic islets were isolated by collagenase digestion method (Okamoto 1981) from male Wistar rats (250-350 g) (SLC, Hamamatsu, Japan), which were fed *ad libitum*. Following the isolation, the islets were incubated for 12-18 h in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS) in the presence or absence of 15-50 U/ml recombinant mouse IL-1β (Sigma, MO, USA). Nicotinamide (Wako Pure Chemical Industries Ltd., Osaka, Japan), 3AB (gifts from Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), 1,5-dihydroxyisoquinoline (1,5-DHIQ) (supplied by Kissei Pharmaceutical Co., Ltd., Matsumoto, Japan) or NMMA monoacetate (Calbiochem-Novabiochem,, CA, USA) was added into the incubation medium 1 h before IL-1β. Incubations were performed under an atmosphere of 95% air and 5% CO_2 at 37°C.

Measurement of Nitrite – Nitrite was analyzed by the method of Green et al. (Green et al. 1982) with minor modification. After the 12 h-incubation in RPMI-1640 (without phenol red) supplemented with 10 % FCS, the medium samples were collected and centrifuged at 8,000 g at 4°C for 10 min. The supernatants (33.8 μl) and 0.9% NaCl (33.8 μl) were added to 7.5 μl of reagent containing 0.5% naphthylethylenediamine dihydrochloride (Wako), 5% sulfanilamide (Wako) and 25% H_3PO_4. After 15-min incubation at 60°C, the absorbance at 546 nm was measured by a model DU-65 spectrophotometer (Beckman, CA, USA).

Measurement of Total Protein Biosynthesis – After the 12 h-culture, the islets were washed twice in Krebs-Ringer bicarbonate buffer containing 0.2% bovine serum albumin (Okamoto 1981) and 11.1 mM glucose. After 15-min incubation in the same buffer under an atmosphere of 95% O_2 and 5% CO_2 at 37°C with shaking, the islets were then incubated for a further 1 h in the buffer containing 11.1 mM glucose and L-[4,5-^{3}H]-leucine (37 MBq/ml) (Amersham, Backingham, UK) under the same condition.

The islets were subsequently washed with Hank's solution and sonicated in redistilled water. Total protein biosynthesis was estimated by measurement of radioactivity by liquid scintillation after trichloroacetic acid precipitation of a fraction of the homogenate.

Polymerase Chain Reaction (PCR) of Reverse-transcripted mRNA – Total RNA was extracted from the islets as described (Kato et al. 1994) after the 12-h incubation. Total RNA (100 ng) was reverse-transcripted into cDNA at 42°C for 1 h in 20 μl of reverse transcriptase buffer (50 mM Tris-HCl (pH. 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT) containing 200 units of Superscript^{TM}, 0.5 mM of dNTP, 1.1 units/μl of RNase inhibitor (Takara Shuzo, Ohtsu, Japan) and 1.5 ng/μl of oligo$(dT)_{12\text{-}18}$ (Amersham). The reverse-transcripted sample (1 μl) was used for PCR amplifications. The PCR was performed in 50 μl of PCR buffer (10 mM Tris-HCl (pH. 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin) containing 0.2 mM of dNTP, 1.25 units of Taq polymerase (Perkin-Elmer, Norwalk, CT, USA) and 25 pmol of the following oligonucleotide primer sets. The sequences of the primers for: iNOS cDNA amplification were 5'-CGTGTGCCTGCTG-CCTTCCTGCTGT-3' and 5'-GTAATCCTCAACCTGCTCCTCACTC-3' (nucleotides 2679 to 2703 and 3326 to 3350 in Ref. 36); those for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA amplification were 5'-CATTGACCTCAACTACATGGT-3' and 5'-TTGTCATACCAGGAAATGAGC-3' (nucleotides 135 to 155 and 951 to 971 in Ref. 46); and those for IRF-1 cDNA amplification were 5'-CTTCAGAGCTTAGG-AGGCAGGGTCT-3' and 5'-AGCAGGCACAGGGCAAGGCACTATA-3' (nucleotides 1356 to 1380 and 1855 to 1879 in Ref. 52), respectively. All the oligodeoxyribonucleotides were synthesized with a model 392 automated DNA synthesizer (Applied Biosystems). PCR was performed in a thermal cycler (model PJ1000; Perkin-Elmer, Norwalk, CT, USA) for 30 cycles for iNOS and GAPDH; for 28 cycles for IRF-1 with the following parameters : denaturation at 94°C for 30 sec, annealing at 62°C for 1 min, and extension at 72°C for 2 min. The PCR products (18 μl)

were electrophoresed in 1.0% Sea Kem GTG™ agarose containing ethidium bromide (0.5 μg/ml).

Measurement of ATP Concentrations in the Islets – After the 18 h-culture of 30 islets, the islets were washed twice in Krebs-Ringer bicarbonate buffer (Okamoto 1981) containing 0.2% bovine serum albumin and 2.7 mM glucose. After 30-min incubation in the same buffer containing 2.7 mM glucose under an atmosphere of 95% O_2 and 5% CO_2 at 37°C with shaking, the islets were then incubated for a further 30 min in the buffer containing 20 mM glucose. Thirty islets were sonicated in 200 μl of ice-cold 8% (vol/vol) perchloric acid containing 2 mM EDTA and immediately plunged into dry ice-cold ethanol. The sonicated samples were neutralized with 1.5 N NaOH and diluted 100 times with 0.1 M Tris-acetate buffer (pH. 7.75) supplemented with 2 mM EDTA. The ATP concentrations of the samples were measured by a bioluminescence assay procedure using an ATP monitoring kit (Bio Orbit, Turku, Finland). The fluorescence intensities were measured by a model 1251 luminometer (Bio Orbit).

Measurement of Secreted Insulin – After the 18 h-culture of 20-30 islets, the islets were washed twice in Krebs-Ringer bicarbonate buffer (Okamoto 1981) containing 0.2% bovine serum albumin and 2.7 mM glucose. After 30-min incubation in the same buffer containing 2.7 mM glucose under an atmosphere of 95% O_2 and 5% CO_2 at 37°C with shaking, the buffer was collected and stored at -30°C. The islets were then incubated for further 30 min in the buffer containing 20 mM glucose and the buffer was stored at -30°C. Determinations of secreted insulin in the buffer were made by an insulin radioimmunoassay kit (Amersham) and rat insulin standard.

RESULTS

Effects of PARS Inhibitors and NMMA on Nitrite Formation – To investigate the effect of the PARS inhibitors and NMMA on IL-1β-induced NO formation, isolated rat pancreatic islets were incubated with IL-1β (15 U/ml) in the presence or absence of various PARS inhibitors or NMMA for 12 h. NO production during the culture was monitored by the accumulated nitrite level in the medium, which reflects the amount of NO produced during the culture. The nitrite levels from the IL-1β-stimulated islets were about 3-fold higher than control levels (Fig. 1A), indicating that IL-1β induces NO production in the pancreatic islets. NMMA (2 mM) completely inhibited nitrite formation. High-concentration nicotinamide (20 mM), but not low-concentration nicotinamide (5 mM), completely inhibited IL-1β-induced nitrite formation. However, 3AB (20 mM) and 1,5-DHIQ (100 μM) failed to inhibit nitrite formation. As shown in Fig. 1B, nicotinamide inhibited IL-1β-induced nitrite formation in a dose-dependent manner.

Effects of PARS Inhibitors on Total Protein Biosynthesis – To assess the effects of PARS inhibitors on total protein synthesis, the protein biosynthesis in the islets was determined by measuring [^{3}H]-leucine incorporation into acid-insoluble protein after 12-h incubation with IL-1β or PARS inhibitors or with both IL-1β and PARS inhibitor (Table I). The exposure of islets to high-concentration nicotinamide (20 mM) alone did not affect the total protein biosynthesis. After the treatment of IL-1β (15 U/ml) plus nicotinamide (20 mM), there was a 16 ± 7 % decrease in the total protein biosynthesis compared with the control. In contrast, the treatment of IL-1β (15 U/ml) plus 3AB (20 mM) decreased the protein synthesis by 44 ± 5 %.

Effects of Nicotinamide on iNOS mRNA Expression – To elucidate the mechanism of high-concentration nicotinamide (20 mM)-mediated prevention of NO production, I next determined the iNOS mRNA levels by reverse-transcripted PCR (Fig. 2). The iNOS

mRNA was strongly expressed in the islets exposed to IL-1β (15 U/ml) for 12 h, but not in the control islets. The high concentration of nicotinamide (20 mM) but not the low concentration of nicotinamide (5 mM) almost completely prevented IL-1β-stimulated iNOS mRNA expression. The GAPDH mRNA, an internal control, was equally expressed under the respective conditions.

Effects of Nicotinamide on IRF-1 mRNA Expression – Since transcriptional factor IRF-1 is essential for iNOS gene expression, I next determined the IRF-1 mRNA level at 1 h after the exposure to IL-1β (15 U/ml) by reverse-transcripted PCR. A high concentration of nicotinamide (20 mM) but not a low concentration of nicotinamide (5 mM) completely inhibited IL-1β-induced IRF-1 mRNA expression (Fig. 3). GAPDH mRNA was equally expressed under the respective conditions.

Effects of Nicotinamide on Intracellular ATP Formation – Since the intracellular ATP formation in pancreatic β cells is crucial for the glucose-induced insulin secretion (Ashcroft 1988; Takasawa et al. 1993b), I measured the ATP concentrations in pancreatic islets after the exposure to high glucose (20 mM). After the 18-h incubation with IL-1β (50 U/ml) or with IL-1β (50 U/ml) and nicotinamide (5 mM or 20 mM), the islets were exposed to 20 mM glucose for 30 min and then assayed for the ATP concentrations using a luminescence assay. As shown in Fig. 4, the IL-1β treatment reduced the intracellular ATP concentration to about 50 % of the control level. In contrast, low-concentration nicotinamide (5 mM) partially, and high-concentration nicotinamide (20 mM) completely, restored the ATP level. NMMA also completely restored the ATP level.

Effects of PARS Inhibitors and NMMA on Glucose-induced Insulin Secretion – After 18-h culture with mouse recombinant IL-1β (50 U/ml) alone or with IL-1β (50 U/ml) and PARS inhibitor or NMMA, islets were incubated for 30 min in the Krebs-Ringer

bicarbonate buffer containing 2.7 mM glucose and then for further 30 min in the buffer containing 20 mM glucose. The secreted insulin levels were then determined by radioimmunoassay. As shown in Fig. 5, the IL-1β-stimulation of rat pancreatic islets resulted in an inhibition of glucose-induced insulin secretion. Low-concentration nicotinamide (5 mM) partially attenuated IL-1β-evoked inhibition of insulin secretion at high glucose condition (20 mM). High-concentration nicotinamide (20 mM) almost completely attenuated the inhibition of insulin secretion at high glucose. However, 3AB (5 and 20 mM) and 1,5-DHIQ (100 μM), which completely inhibit poly(ADP-ribosyl)ation at these concentrations (Banasik et al. 1992), failed to attenuate the inhibition of insulin secretion. NMMA (2 mM), a NOS inhibitor, completely attenuated the inhibition of insulin secretion.

DISCUSSION

The nitrite levels in the medium of IL-1β-treated islets were about 3-fold higher than the control levels (Fig. 1A), indicating that IL-1β induces NO production in pancreatic islets. NMMA (2 mM) completely inhibited nitrite formation, indicating that this nitrite accumulation was indeed derived from NO synthase. High-concentration nicotinamide (20 mM) completely inhibited IL-1β-induced nitrite formation. Low-concentration nicotinamide (5 mM), which almost completely inhibits PARS activity (Yamamoto and Okamoto 1980), did not significantly inhibited IL-1β-induced nitrite formation. 3AB (20 mM) and 1,5-DHIQ (100 μM), which also completely inhibit PARS activity (Banasik et al. 1992), failed to inhibit nitrite formation.

The iNOS mRNA was strongly expressed in the islets exposed to IL-1β, but not in the control islets (Fig. 2). The high concentration of nicotinamide (20 mM) but not the low concentration of nicotinamide (5 mM) almost completely prevented IL-1β-stimulated iNOS mRNA expression, indicating that the inhibitory effects of nicotinamide (20 mM) on IL-1β-induced NO formation is primarily based on the reduction of the iNOS mRNA level. The GAPDH mRNA, an internal control, was equally expressed under the respective conditions; this suggests that high-concentration nicotinamide does not exert its effect via a general suppression of RNA synthesis. In addition, [^{3}H]-leucine incorporation into the islets was not changed by incubation with nicotinamide (up to 20 mM), indicating that nicotinamide does not affect total protein synthesis (Table I). Cetkovic-Cvrlje et al. reported that nicotinamide (10 and 20 mM) inhibits IL-1-induced NO production in RINm5F cells without decreasing iNOS mRNA expression (Cetkovic-Cvrlje et al. 1993), but RINm5F cells, a line of tumorous islet cells, are known to differ from normal islet cells in many biochemical and functional respects (Malaisse 1990). Therefore, the results in this study seem to reflect events under a physiological condition.

The promoter sequence of the murine iNOS gene (Xie et al. 1993) contains cytokine-responsive elements for the binding of transcriptional factors such as nuclear

factor κB (NFκB) and IRF-1 (Miyamoto et al. 1988). It has been suggested that NFκB activation is required for IL-1β-induced iNOS mRNA expression (Saldeen and Welsh 1994; Bedoya et al. 1995). However, the recent study using IRF-1 knock-out mice indicated that IRF-1 is absolutely essential for iNOS mRNA induction (Kamijo et al. 1994). I therefore determined the IRF-1 mRNA level (at 1 h after the exposure to IL-1β) and found that a high concentration of nicotinamide (20 mM) but not a low concentration of nicotinamide (5 mM) completely inhibited IL-1β-induced IRF-1 mRNA expression (Fig. 3). The IRF-1 mRNA is immediately induced when the cells are activated by various stimuli such as cytokines and degradates within a few hours (Fujita et al. 1989). Thus, the inhibition of iNOS mRNA induction by high-concentration nicotinamide was thought to be achieved through the inhibition of IRF-1 mRNA induction.

It has been suggested that NO inhibits iron-containing enzymes such as aconitase and complexes I and II by destroying their iron-sulfur centers (Corbett et al. 1992). Since aconitase and complexes I and II, which are involved in Krebs cycle and electron transport respectively, play important roles for ATP generation in mitochondria, the ATP reduction in the IL-1β-treated islets was expected. As shown in Fig. 4, the IL-1β treatment reduced the intracellular ATP concentration to about 50 % of the control. In contrast, low-concentration nicotinamide (5 mM) partially, and high-concentration nicotinamide (20 mM) completely, restored the ATP level. NMMA also completely restored the ATP level, indicating that IL-1β-evoked ATP depletion is indeed mediated by NO.

As shown in Fig. 5, the IL-1β-stimulation of isolated pancreatic islets resulted in the inhibition of glucose-induced insulin secretion. Low-concentration nicotinamide (5 mM) partially, and high-concentration nicotinamide (20 mM) almost completely, attenuated the inhibition of insulin secretion at high glucose. Similar effects of high-concentration nicotinamide have been reported (Buscema et al. 1992; Andersen et al. 1994; Eizirik et al. 1994). However, 3AB (5 and 20 mM) and 1,5-DHIQ (100 μM),

which completely inhibit poly(ADP-ribosyl)ation at these concentrations (Banasik et al. 1992), failed to attenuate the inhibition of insulin secretion. This suggests that the attenuation by high-concentration nicotinamide can not be simply explained by the inhibition of poly(ADP-ribosyl)ation. Since NMMA, a NO synthase inhibitor, completely attenuated the inhibition of insulin secretion (Fig. 5), the inhibitory effect of IL-1β is mediated by NO. NO has been also reported to mediate cytokine-induced inhibition of insulin secretion in human islets (Corbett et al. 1993). Although Rabinovitch et al. reported that human islet β cell destruction by cytokines was independent of NO production (Rabinovitch et al. 1994), they incubated human single β cells for a long time (84 h) in combination of cytokines.

Takasawa et al. have suggested that cyclic ADP-ribose plays a second messenger role in the glucose-induced insulin secretion by mobilizing Ca^{2+} from intracellular Ca^{2+} stores (Takasawa et al. 1993a). In fact, intracellular Ca^{2+} elevation by glucose stimulation in the absence of extracellular Ca^{2+} has been recently confirmed in pancreatic β cells (Rojas et al. 1994). Takasawa et al. have proposed that ATP, generated in the course of glucose metabolism, plays a critical role in the glucose-induced insulin secretion by increasing cyclic ADP-ribose (Takasawa et al. 1993b). ATP also regulates closure/opening of ATP-sensitive K^+ channels, which play a critical role in cell membrane depolarization, resulting in Ca^{2+}-influx (Ashcroft 1988). Ashcroft et al. have reported that the islet ATP content increases from 6 to 7.5 mM when glucose concentration was rised from 3 to 20 mM (Stephen et al. 1973). Therefore, 50 % decrease of intracellular ATP content in IL-1β-stimulated islets (Fig. 4) may explain dramatic inhibition of glucose-induced insulin secretion. Therefore, the recovery of ATP concentrations by high-concentration nicotinamide may explain why this agent restores glucose-induced insulin secretion (Fig. 5).

From the results of the present study, the possible mechanisms by which nicotinamide prevents IL-1β-induced β cell dysfunction are also schematically shown in Fig. 6: (i) low-concentration nicotinamide (5 mM) prevents the IL-1β-evoked islet cell

death by restoring cellular NAD^+ via the inhibition of PARS activation, but can not completely restore β cell functions such as insulin secretion, because cellular ATP is reduced by NO; (ii) high-concentration nicotinamide (20 mM) restores β cell functions by maintaining both NAD^+ and ATP levels because it prevents not only PARS activation but also iNOS gene expression by inhibiting IRF-1 mRNA induction.

Since administration of a NO synthase inhibitor such as NMMA *in vivo* decreases NO generation, NMMA causes hypertension by increasing peripheral vascular resistance (Haynes et al. 1993). In contrast, no serious toxic effects of nicotinamide have been reported in human. Some clinical studies in diabetic patients showed that oral administration of nicotinamide protects residual β cell function including insulin secretion (Vague et al. 1989), and prevents IDDM (Elliott and Chase 1991; Pozzilli et al. 1995). In these cases, a concentration of nicotinamide in the serum was estimated to be about 2-4 mM. Large doses of nicotinamide have been also reported to have preventive effect on diabetes in nonobese diabetic mice, a model of IDDM (Yamada et al. 1982). In this case, the concentration of nicotinamide was estimated to be 16-32 mM. The present results establish the molecular basis of the preventive effect of high-dose nicotinamide on IDDM and emphasize the importance of the dose of nicotinamide used in the treatment of IDDM patients.

REFERENCES

1) Andersen, H.U., Jørgensen, K.H., Egeberg, J., Mandrup-Poulsen, T. & Nerup, J. (1994) Nicotinamide prevents interleukin-1 effects on accumulation insulin release and nitric oxide production in rat islets of Langerhans. *Diabetes*, **43**, 770-777.

2) Ankarcrona, M., Dypbukt, J.M., Brüne, B. & Nicotera, P. (1994) Interleukin-1β-induced nitric oxide production activates apoptosis in pancreatic RINm5F cells. *Exp. Cell Res.*, **213**, 172-177.

3) Ashcroft, F.M. (1988) Adenosine 5'-triphosphate-sensitive potassium channels. *Annu. Rev. Neurosci.*, **11**, 97-118.

4) Ashcroft, S.J.H., Weerasinghe, L.C.C. & Randle, P.J. (1973) Interrelationship of islet metabolism, adenosine triphosphate content and insulin release. *Biochem. J.*, **132**, 223-231.

5) Banasik, M., Komura, H., Shimoyama, M. & Ueda, K. (1992) Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribose) transferase. *J. Biol. Chem.*, **267**, 1569-1575.

6) Bedoya, F.J., Flodström, M. & Eizirik, D.L. (1995) Pyrrolidine dithiocarbamate prevents IL-1-induced nitric oxide synthase mRNA, but not superoxide dismutase mRNA, in insulin producing cells. *Biochem. Biophys. Res. Commun.*, **210**, 816-822.

7) Bolaffi, J.L., Rodd, G.G., Wang, J. & Grodsky, G.M. (1994) Interrelationship of changes in islet nicotinamide adeninedinucleotide, insulin secretion, and cell viability induced by interleukin-1β. *Endocrinology*, **134**, 537-542. 1994

8) Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R. & Snyder S.H. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature*, **351**, 714-718.

9) Buscema, M., Vinci, C., Gatta, C., Rabuazzo, M.A., Vignen, R. & Purrello, F. (1992) Nicotinamide partially reverses the interleukin-1β inhibition of glucose-induced insulin release in pancreatic islets. *Metabolism*, **41**, 296-300.

10) Cetkovic-Cvrlje, M., Sandler, S. & Eizirik, D.L. (1993) Nicotinamide and dexamethasone inhibit interleukin-1-induced nitric oxide production by RINm5F cells without decreasing messenger ribonucleic acid expression for nitric oxide synthase. *Endocrinology*, **133**, 1739-1743.

11) Cooke, A. (1990) An overview on possible mechanisms of destruction of the insulin-producing beta cell. *Curr. Top. Microbiol. Immunol.*, **164**, 125-142.

12) Corbett, J.A., Lancaster, J.R., Jr., Sweetland, M.A. & McDaniel, M.L. (1991) Interleukin-1β-induced formation of EPR-detectable iron-nitrosyl complexes in islets of Langerhans. *J. Biol. Chem.*, **266**, 21351-21354.

13) Corbett, J.A., Wang, J.L., Sweetland, M.A., Lancaster, J.R., Jr. & McDaniel, M.L. (1992) Interleukin 1β induces the formation of nitric oxide by β-cells purified from rodent islets of Langerhans. *J. Clin. Invest.*, **90**, 2384-2391.

14) Corbett, J.A., Sweetland, M.A., Wang, J.L., Lancaster Jr, J.R. & McDaniel, M.L. (1993) Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. *Proc. Natl. Acad. Sci. USA*, **90**, 1731-1735.

15) Delaney, C.A., Green, M.H.L., Lowe, J.E. & Green, I.C. (1993) Endogenous nitric oxide induced by interleukin-1β in rat islets of Langerhans and HIT-T15 cells causes significant DNA damage as measured by the 'comet' assay. *FEBS Lett.*, 333, 291-295.

16) Eizirik, D.L., Sandler, S., Welsh, N., Bendtzen, K. & Hellerström, C. (1994) Nicotinamide decreases nitric oxide production and partially protects human pancreatic islets against the suppression effects of combinations of cytokines. *Autoimmunity*, **19**, 193-198.

17) Elliott, R.B. & Chase, H.P. (1991) Prevention or delay of type 1 (insulin-dependent) diabetes mellitus in children using nicotinamide. *Diabetologia*, **34**, 362-365.

18) Fujita, T., Reis, L.F.L., Watanabe, N., Kimura, Y., Taniguchi, T. & Vilcek, J. (1989) Induction of the transcription factor IRF-1 and interferon-β mRNAs by cytokines

and activators of second-messenger pathways. *Proc. Natl. Acad. Sci. USA*, **86**, 9936-9940.

19) Gepts, W. (1965) Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes*, **14**, 619-633.

20) Green, I.C., Cunningham, J.M., Delaney, C.A., Elphick, M.R., Mabley, J.G. & Green, M.H.L. (1994) Effects of cytokines and nitric oxide donors on insulin secretion, cyclic GMP and DNA damage: relation to nitric oxide production. *Biochem. Soc. Trans.*, **22**, 30-37.

21) Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. & Tannenbaum, S.R. (1982) Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131-138.

22) Haynes, W.G., Noon, J.P., Walker, B.R. & Webb, D.J. (1993) Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. *J. Hypertension*, **11**, 1375-1380.

23) Heller, B., Wang, Z-Q., Wagner, E.F., Radons, J., Bürkle, A., Fehsel, K., Burkart, V. & Kolb, H. (1995) Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. *J. Biol. Chem.*, **270**, 11176-11180.

24) Janssens, S.P., Shimoushi, A., Quertermous, T., Bloch, D.B. & Blach, K.D. (1992) Cloning and expression of a cDNA encording human endothelium-derived relaxing factor/nitric oxide synthase. *J. Biol. Chem.*, **267**, 14519-14522

25) Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Koh, S.I., Kimura, T., Green, S.J., Mak, T.W., Taniguchi, T. & Vilcek, J. (1994) Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science*, **263**, 1612-1615.

26) Kato, I., Suzuki, Y., Akabane, A., Yonekura, H., Tanaka, O., Kondo, H., Takasawa, S., Yoshimoto, T. & Okamoto, H. (1994) Transgenic mice overexpressing human vasoactive intestinal peptide (VIP) in pancreatic β cells. Evidence for improved

glucose tolerance and enhanced insulin secretion by VIP and PHM-27 *in vivo*. *J. Biol. Chem.*, **269**, 21223-21228.

27) Malaisse, W.J. (1990) in *Molecular Biology of the Islets of Langerhans* (Okamoto, H., ed) pp.315-339. Cambridge University Press, Cambridge, United Kingdom.

28) Mandrup-Poulsen, T., Bendtzen, K., Nielsen, J.H., Bendixen, G. & Nerup, J. (1985) Cytokines cause functional and structural damage to isolated islets of Langerhans. *Allergy (Cph.)*, **40**, 424-429.

29) Mandrup-Poulsen, T., Bendtzen, K., Nerup, J., Egeberg, J. & Nielsen, J.H. (1986) Mechanisms of pancreatic islet-cell destruction: dose-dependent cytotoxic effect of soluble blood mononuclear cell mediators on isolated islets of Langerhans. *Allergy (Cph.)*, **41**, 250-259.

30) Mandrup-Poulsen, T., Helqvist, S., Wogensen, L.D., Molvig, J., Pociot, F., Johannesen, J. & Nerup, J. (1990) Cytokines and free radicals as a effector molecules in the destruction of pancreatic beta cells. *Curr. Top. Microbiol. Immunol.*, **164**, 169-193.

31) Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T. & Taniguchi, T. (1988) Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-β gene regulatory elements. *Cell*, **54**, 903-913.,

32) Moncada, S., Palmer, R.M. & Higgs, E.A. (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109-142.

33) Moncada, S. (1992) The L-arginine: nitric oxide pathway. *Acta Physiol. Scand.*, **145**, 201-227.

34) Nathan, C. & Hibbs, J.B., Jr. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.*, **3**, 65-70.

35) Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J.*, **6**, 3051-3064.

36) Nunokawa, Y., Ishida, N. & Tanaka., S. (1993) Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **191**, 89-94.

37) Okamoto, H. (1981) Regulation of proinsulin synthesis in pancreatic islets and a new aspect to insulin-dependent diabetes. *Mol. Cell. Biochem.*, **37**, 43-61.

38) Okamoto, H. (1990) in *Molecular Biology of the Islets of Langerhans* (Okamoto, H., ed) pp.209-231, Cambridge University Press, Cambridge, United Kingdom.

39) Pozzilli, P., Visalli, N., Signore, A., Baroni, M.G., Buzzetti, R., Cavallo, M.G., Boccuni, M.L., Fava, D., Gragnoli, C., Andreani, D., Lucentini, L., Matteoli, M.C., Crinò, A., Cicconetti, C.A., Teodonio, C., Paci, F., Amoretti, R., Pisano, L., Pennafina, M.G., Santopadre, G., Marozzi, G., Multari, G., Suppa, M.A., Campea, L., De Mattia, G.C., Cassone Faldetta, M., Marietti, G., Perrone, F., Greco, A.V. & Ghirlanda, G. (1995) Double blind trial of nicotinamide in recent-onset IDDM. *Diabetologia*, **38**, 848-852.

40) Rabinovitch, A., Suarez-Pinzon, W.L., Strynadka, K., Schulz, R., Lakey, J.R.T., Warnock, G.L. & Rajotte, R.V. (1994) Human pancreatic islet β-cell destruction by cytokines is independent of nitric oxide production. *J. Clin. Endocr. Metab.*, **79**, 1058-1062.

41) Rojas, E., Carroll, P.B., Ricordi, C., Boschero, A.C., Stojilkovic, S.S. & Atwater, I. (1994) Control of cytosolic free calcium in cultured human pancreatic β-cells occurs by external calcium-dependent and independent mechanisms. *Endocrinology*, **134**, 1771-1781.

42) Saldeen, J. & Welsh, N. (1994) Interleukin-1β induced activation of NF-κB in insulin producing RINm5F cells is prevented by the protease inhibitor Nα-p-tosyl-L-lysine chloromethylketone. *Biochem. Biophys. Res. Commun.*, **203**, 149-155.

43) Sandler, S., Andersson, A. & Hellerström, C. (1987) Inhibitory effects of interleukin 1 on insulin secretion, insulin biosynthesis, and oxidative metabolism of isolated rat pancreatic islets. *Endocrinology*, **121**, 1424-1431.

44) Takasawa, S., Nata, K., Yonekura, H. & Okamoto, H. (1993a) Cyclic ADP-ribose in insulin secretion from pancreatic β cells. *Science*, **259**, 370-373.

45) Takasawa, S., Tohgo, A., Noguchi, N., Koguma, T., Nata, K., Sugimoto, T., Yonekura, H. & Okamoto H (1993b) Synthesis and hydrolysis of cyclic ADP-ribose by human leukocyte antigen CD38 and inhibition of the hydrolysis by ATP. *J. Biol. Chem.*, **268**, 26052-26054.

46) Tso, J.Y., Sun, X-H., Kao, T-h., Reece, K.S. & Wu, R. (1985) Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.*, **13**, 2485-2502.

47) Uchigata, Y., Yamamoto, H., Kawamura, A. & Okamoto, H. (1982) Protection by superoxide dismutase, catalase, and poly(ADP-ribose) synthetase inhibitors against alloxan- and streptozotocin-induced islet DNA strand breaks and against the inhibition of proinsulin synthesis. *J. Biol. Chem.*, **257**, 6084-6088.

48) Vague, P., Picq, R., Bernal, M., Lassmann-Vague, V. & Vialettes, B. (1989) Effect of nicotinamide treatment on the residual insulin secretion in type 1 (insulin-dependent) diabetic patients. *Diabetologia*, **32**, 316-321.

49) Yamada, K., Nonaka, K., Hanafusa, T., Miyazaki, A., Toyoshima, H. & Tarui, S. (1982) Preventive and therapeutic effects of large-dose nicotinamide injections on diabetes associated with insulitis. *Diabetes*, **31**, 749-753.

50) Yamamoto, H.& Okamoto, H. (1980) Protection by picolinamide, a novel inhibitors of poly(ADP-ribose) synthetase, against both streptozotocin-induced depression of proinsulin synthesis and production of NAD content in pancreatic islets. *Biochem. Biophys. Res. Commun.*, **95**, 474-481.

51) Yamamoto, H., Uchigata, Y. & Okamoto, H. (1981) Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. *Nature*, **294**, 284-286.

52) Yu-Lee, L.Y., Hrachovy, J.A., Stevens, A.M. & Schwarz, L.A. (1990) Interferon-regulatory factor-1 is an immediate-early gene under transcriptional regulation by prolaction in Nb2 T cells. *Mol. Cell. Biol.*, **10**, 3087-3094.

53) Xie, Q-W., Whisnant, R. & Nathan, C. (1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon g and bacterial lipopolysaccharide. *J. Exp. Med.*, **177**, 1779-1784.

FIGURE LEGENDS

FIG. 1A **Effects of PARS inhibitors and NMMA on nitrite formation from IL-1β-treated islets.** Isolated islets were incubated at 37°C in the medium alone (RPMI-1640 + 10 % FCS) (*Control*), or the medium containing 15 U/ml IL-1β (*IL-1β*), IL-1β + 5 mM nicotinamide (*IL-1β + 5 mM NA*), IL-1β + 20 mM nicotinamide (*IL-1β + 20 mM NA*), IL-1β + 20 mM 3AB (*IL-1β + 3AB*), IL-1β + 100 μM 1,5-DHIQ (*IL-1β + 1,5-DHIQ*) or IL-1β + 2 mM NMMA (*IL-1β + NMMA*). After 12-h incubation, nitrite formation in the medium was determined by the method of Green et al. (Green et al. 1982) with minor modification. N≥4 for each condition. *, P<0.01. *Vertical bars* indicate standard errors of the mean (SEM). Statistical analyses were performed using student's *t* test.

FIG. 1B. **Dose-dependency of nicotinamide on the inhibition of nitrite formation from IL-1β-treated islets.** Isolated islets were incubated in the presence of 15 U/ml IL-1β and in the presence or absence of various concentrations of nicotinamide. After 12-h incubation, nitrite formation in the medium was determined by the method of Green et al. with minor modification. Nitrite levels were expressed as % of those stimulated with IL-1β alone. N≥2 for each condition.

FIG. 2. **Effects of nicotinamide on the expressions of iNOS mRNA in IL-1β-treated islets.** Isolated islets were incubated at 37°C in the medium alone (RPMI-1640 + 10 % FCS) (*Control*), or the medium containing 15 U/ml IL-1β (*IL-1β*), IL-1β + 5 mM nicotinamide (*IL-1β + 5 mM NA*) or IL-1β + 20 mM nicotinamide (*IL-1β + 20 mM NA*). After 12-h incubation, the iNOS mRNA and GAPDH mRNA (internal control) expressions were analyzed by reverse-transcripted PCR. Upper panel (*iNOS*) and lower panel (*GAPDH*) show the reverse-transcripted PCR products for iNOS mRNA and GAPDH mRNA, respectively. Two independent experiments gave similar results.

FIG. 3. **Effects of nicotinamide on the expressions of IRF-1 mRNA in IL-1β-treated islets.** Isolated islets were incubated at 37°C in the medium containing 15 U/ml IL-1β (*IL-1β*), IL-1β + 5 mM nicotinamide (*IL-1β + 5 mM NA*) or IL-1β + 20 mM nicotinamide (*IL-1β + 20 mM NA*). After 1-h incubation, the IRF-1 mRNA and GAPDH mRNA (internal control) expressions were analyzed by reverse-transcripted PCR. Upper panel (*IRF-1*) and lower panel (*GAPDH*) show the reverse-transcripted PCR products for IRF-1 mRNA and GAPDH mRNA, respectively. Two independent experiments gave similar results.

FIG. 4. **Effects of nicotinamide and NMMA on IL-1β-induced reduction of intracellular ATP concentrations.** Isolated islets were incubated in the medium alone (RPMI-1640 + 10 % FCS) (*Control*), or the medium containing 50 U/ml IL-1β (*IL-1β*), IL-1β + 5 mM nicotinamide (*IL-1β + 5 mM NA*), IL-1β + 20 mM nicotinamide (*IL-1β + 20 mM NA*) or IL-1β + 2 mM NMMA(*IL-1β + NMMA*). Following 18-h incubation, islets were incubated for 30 min in Krebs-Ringer bicarbonate buffer containing 20 mM glucose. Thirty islets were sonicated and then the ATP concentrations of the samples were measured by a bioluminescence assay procedure. N≥4 for each condition. *, $P < 0.02$; **, $P < 0.01$. *Vertical bars* indicate SEM. Statistical analyses were performed by student's *t* test.

FIG. 5. **Effects of PARS inhibitors and NMMA on the IL-1β-induced inhibition of insulin secretion.** After 18-h incubation of isolated islets in the medium alone (RPMI-1640 + 10 % FCS) (*Control*), or the medium containing 50 U/ml IL-1β (*IL-1β*), IL-1β + 5 mM nicotinamide (*IL-1β + 5 mM NA*), IL-1β + 20 mM nicotinamide (*IL-1β + 20 mM NA*), IL-1β + 5 mM 3AB (*IL-1β + 5 mM 3AB*), IL-1β + 20 mM 3AB (*IL-1β + 20 mM 3AB*), IL-1β + 100 μM 1,5-DHIQ (*IL-1β + 1,5-DHIQ*) or IL-1β + 2 mM NMMA (*IL-1β + NMMA*), 20-30 islets were incubated for 30 min in Krebs-Ringer bicarbonate buffer containing 2.7 mM glucose . The buffer was collected and then the islets were

incubated for further 30 min in the buffer containing 20 mM glucose. Determinations of the insulin levels in the buffer were made by radioimmunoassay. *Open bars* and *shaded bars* show the insulin levels in 2.7 mM and 20 mM glucose, respectively. $N \geq 4$ for each condition. *, $P < 0.01$. *Vertical bars* indicate SEM. Statistical analyses were performed by student's *t* test.

Fig. 6. **The possible mechanisms of low-concentration and high-concentration nicotinamide against IL-1β-induced pancreatic β cell dysfunction and β cell death.** As indicated by the *opened arrow*, low-concentration nicotinamide prevents the IL-1β-evoked islet cell death by restoring cellular NAD^+ via the inhibition on PARS activation, but can not restore β cell functions such as insulin secretion, because generated NO does reduce cellular ATP. As indicated by the *shaded arrow*, high-concentration nicotinamide restores β cell functions by maintaining both NAD^+ and ATP levels because it prevents not only PARS activation but also iNOS mRNA expression by inhibiting IRF-1 mRNA induction.

Table I

Effects of PARS Inhibitors on Total Protein Biosynthesis in the Islets

After the 12 h-culture in the medium alone *(Control)*, or the medium containing 15 U/ml IL-1β, 20 mM nicotinamide, 20 mM 3AB, IL-1β + 20 mM nicotinamide, IL-1β + 20 mM 3AB, the islets were incubated for a further 1 h in the buffer containing 11.1 mM glucose and L-[4,5-^{3}H]leucine (37 MBq/ml) under an atmosphere of 95% O_2 and 5% CO_2 at 37°C with shaking. The islets were subsequently washed with Hank's solution and sonicated in redistilled water. Total protein biosynthesis was estimated by measurement of radioactivity by liquid scintillation after trichloroacetic acid precipitation of a fraction of the homogenate. The protein biosynthesis was expressed as % of control.

Culture Condition	Total Protein Biosynthesis (% of Control)
Control	100
20 mM NA	91
20 mM 3AB	75
IL-1β	81
IL-1β + 20 mM NA	84
IL-1β + 20 mM 3AB	56

Nitrite Formation (pmol/islet·12h)
0
1
2
3
4
5
6
7
8
*
*
Control
IL-1β
IL-1β +5mM NA
IL-1β +20mM NA
IL-1β + 3AB
IL-1β + 1,5-DHIQ
IL-1β + NMMA

Fig. 1A

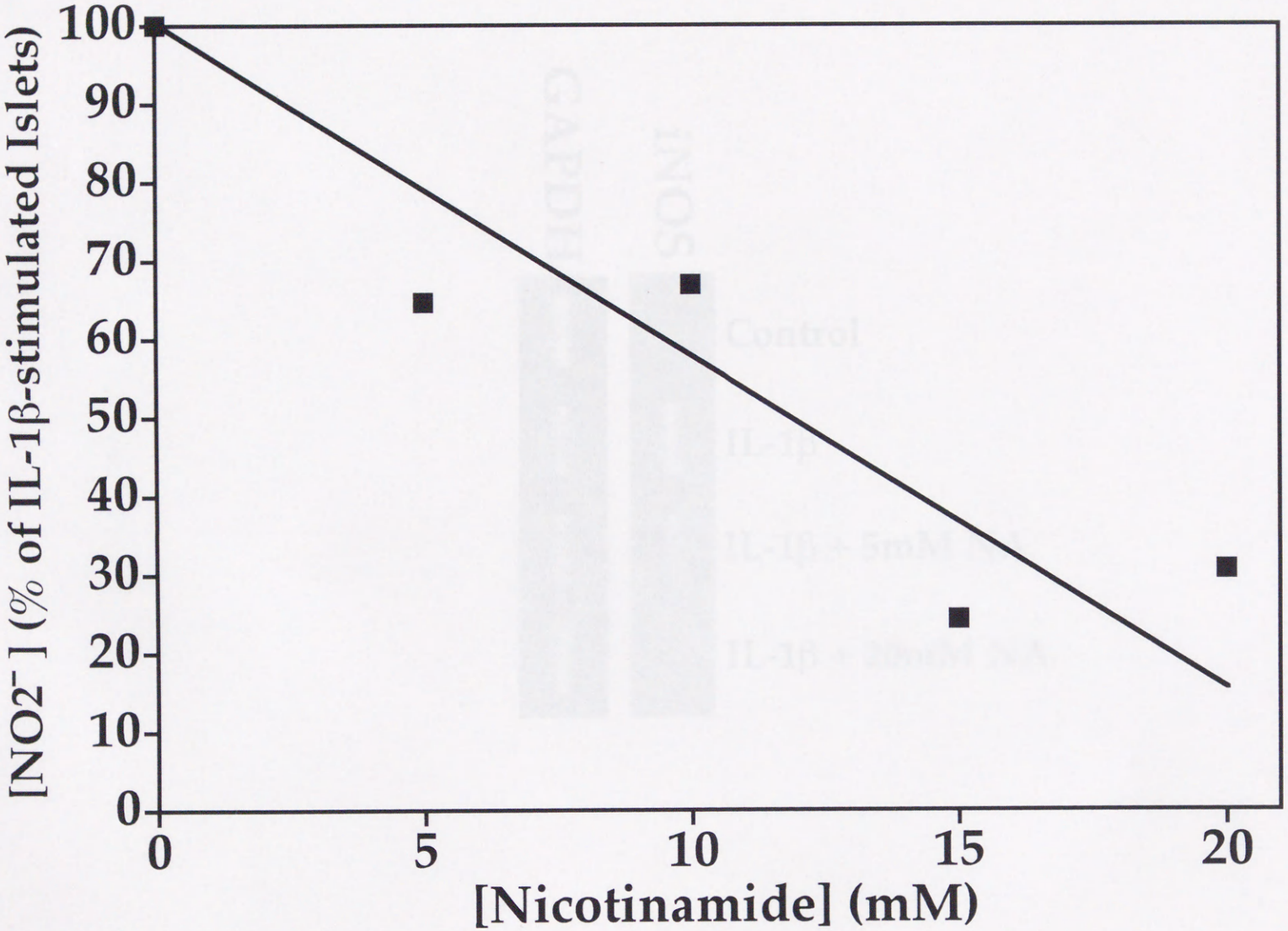

[NO2⁻] (% of IL-1β-stimulated Islets)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
[Nicotinamide] (mM)

Fig. 1B

Fig.2

Fig.3

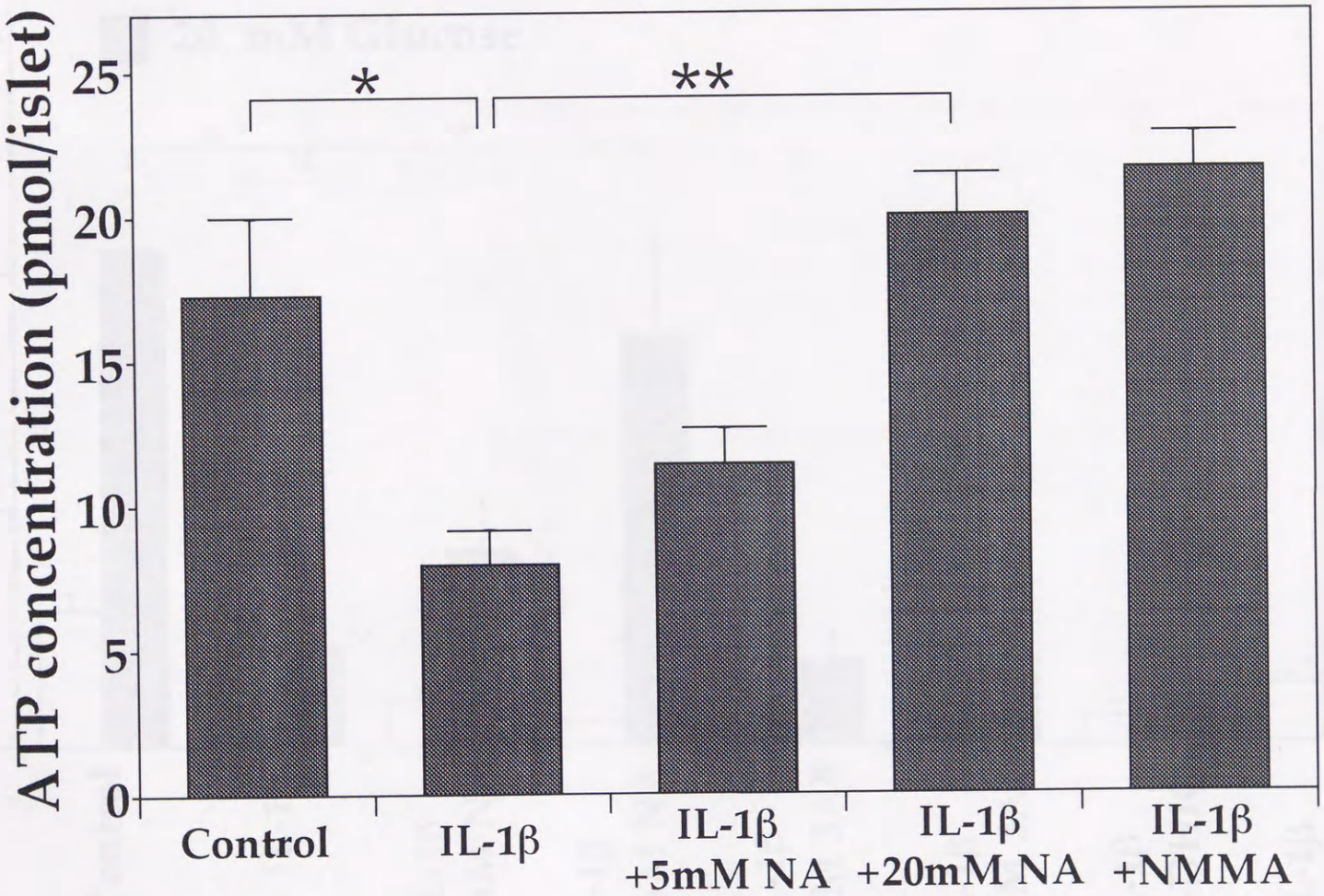

ATP concentration (pmol/islet)
25
20
15
10
5
0
*
**
Control
IL-1β
IL-1β +5mM NA
IL-1β +20mM NA
IL-1β +NMMA

Fig. 4

Secreted Insulin (ng/islets·h)
0
5
10
15
2.7 mM Glucose
20 mM Glucose
*
*
Control
IL-1β
IL-1β + 5mM NA
IL-1β + 20mM NA
IL-1β + 5mM 3AB
IL-1β + 20mM 3AB
IL-1β + 1,5-DHIQ
IL-1β + NMMA

Fig. 5

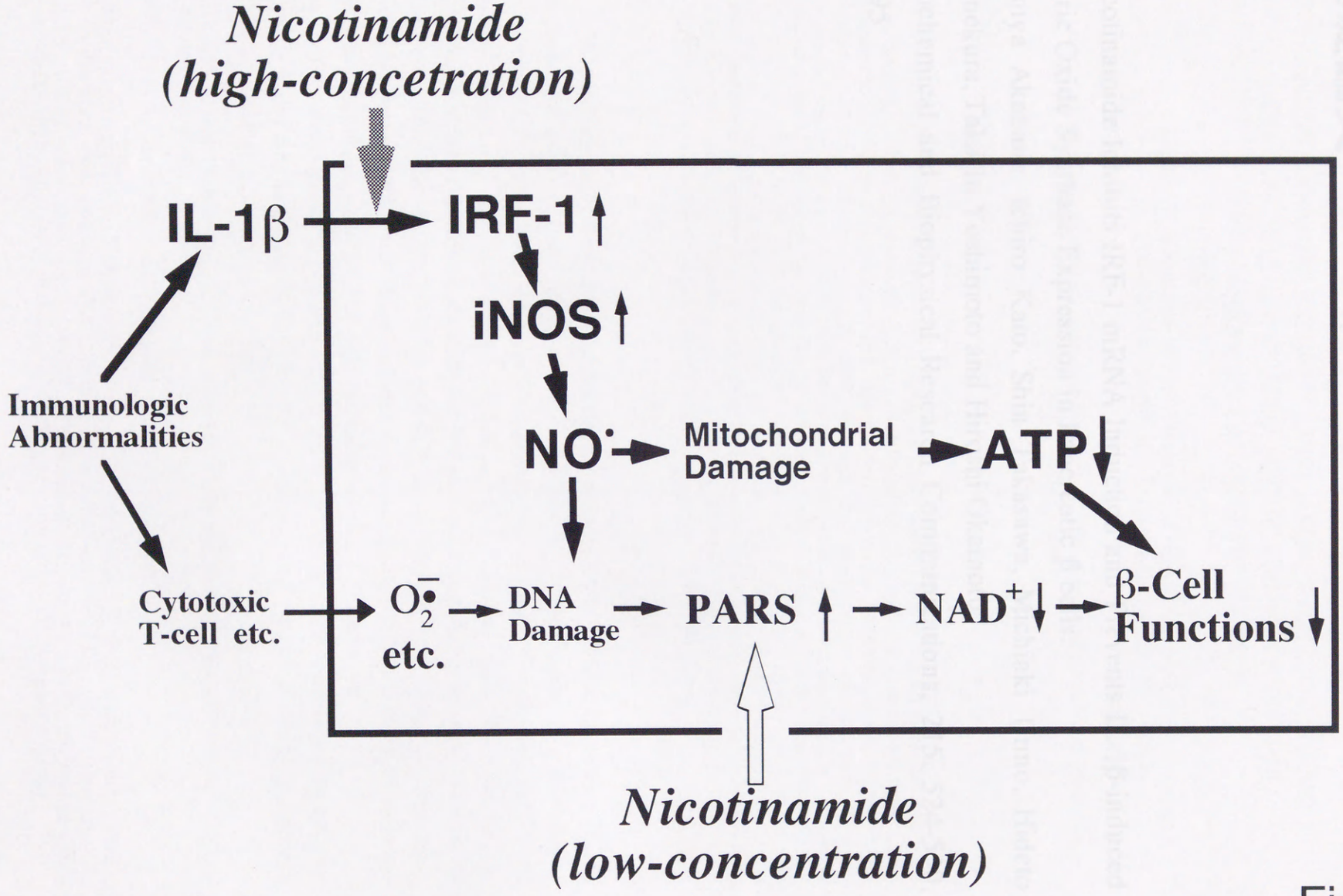

Nicotinamide
(high-concetration)
IL-1β
IRF-1
iNOS
Immunologic
Abnormalities
NO·
Mitochondrial
Damage
ATP
Cytotoxic
T-cell etc.
$O_2^{\bar{}\bullet}$
etc.
DNA
Damage
PARS
NAD^+
β-Cell
Functions
Nicotinamide
(low-concentration)

Fig. 6

基礎論文

Nicotinamide Inhibits IRF-1 mRNA Induction and Prevents IL-1β-induced Nitric Oxide Synthase Expression in Pancreatic β cells.

Atsuya Akabane, Ichiro Kato, Shin Takasawa, Michiaki Unno, Hideto Yonekura, Takashi Yoshimoto and Hiroshi Okamoto.

Biochemical and Biophysical Research Communications, 215, 524-530, 1995

Vol. 215, No. 2, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 13, 1995 Pages 524-530

NICOTINAMIDE INHIBITS IRF-1 mRNA INDUCTION AND PREVENTS IL-1β-INDUCED NITRIC OXIDE SYNTHASE EXPRESSION IN PANCREATIC β CELLS

Atsuya Akabane[1,*], Ichiro Kato[1], Shin Takasawa[1], Michiaki Unno[1], Hideto Yonekura[1], Takashi Yoshimoto[2] and Hiroshi Okamoto[1,§]

Departments of [1]Biochemistry and [2]Neurosurgery, Tohoku University School of Medicine, Sendai 980-77, Miyagi, Japan

Received September 10, 1995

SUMMARY: Nitric oxide produced by inducible nitric oxide synthase in islets exerts inhibitory and cytotoxic effects on pancreatic β cells and is therefore thought to be a potent mediator in the pathogenesis of Type I diabetes mellitus. Here, using isolated rat pancreatic islets, we show that high-concentration nicotinamide (20 mM), but not low-concentration nicotinamide (5 mM), attenuates the interleukin-1β-evoked inhibition of glucose-induced insulin secretion by preventing the induction of interferon regulatory factor-1, a transcriptional factor which plays an essential role in inducible nitric oxide synthase gene expression, and the interleukin-1β-induced nitric oxide formation. High-concentration nicotinamide also restored an interleukin-1β-induced decrease in ATP content in pancreatic β cells, suggesting that interleukin-1β-induced nitric oxide inhibits the mitochondrial function. The present results show the molecular basis of the preventive effect of high-dose nicotinamide on Type I diabetes mellitus.

We have previously found that streptozotocin and alloxan, which produce diabetes mellitus in experimental animals, cause DNA strand breaks which activate nuclear poly(ADP-ribose) synthetase (1-3). The activation of poly(ADP-ribose) synthetase depletes intracellular NAD^+ and inhibits β cell functions such as insulin synthesis, and β cell ultimately dies. Poly(ADP-ribose) synthetase inhibitors such as nicotinamide and 3-aminobenzamide (3AB) reverse the reduction of the NAD^+ level and also the inhibition of insulin biosynthesis (1-3). Recent studies using poly(ADP-ribose) synthetase knock-out mice confirmed that the poly(ADP-ribose) synthetase activation is a major cause of NAD^+ depletion and subsequent β cell death (4).

Interleukin-1β (IL-1β) has been reported to inhibit islet cell functions such as glucose-induced insulin secretion by increasing nitric oxide (NO) formation (5, 6). IL-1β, produced by activated

*Submitted part of this work as partial fulfillment of the degree of Doctor of Medical Science at Tohoku University.

§To whom correspondence should be addressed. Fax 022-272-8101.

Abbreviations: 3AB, 3-aminobenzamide; IL-1β, interleukin-1β; NO, nitric oxide; iNOS, inducible nitric oxide synthase; IRF-1, interferon regulatory factor-1; FCS, fetal calf serum; 1,5-DHIQ, 1,5-dihydroxyisoquinoline; NMMA, N^G-monomethyl-L-arginine; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NFκB, nuclear factor κB.

0006-291X/95 $12.00

macrophages *etc.*, induces inducible nitric oxide synthase (iNOS) in pancreatic islets (7). NO, thus produced by iNOS in islets, exerts inhibitory and cytotoxic effects on pancreatic β cells (7). Recent studies have shown that NO produced in large amounts by iNOS causes DNA strand breaks (8) and NAD^+ depletion (9), leading to islet cell death (5, 10). However, high-concentration nicotinamide (20 mM) but not low-concentration nicotinamide (5 mM) has been reported to attenuate the IL-1β-induced inhibition of glucose-induced insulin secretion (11). In the present study, we showed that high-concentration nicotinamide inhibits the induction of interferon regulatory factor-1 (IRF-1), a transcriptional factor which is essential for iNOS gene expression (12), thereby attenuating the NO-induced β-cell dysfunction.

MATERIALS AND METHODS

Islets isolation and culture: Pancreatic islets of male Wistar rats (250-350 g) (SLC, Hamamatsu, Japan) were isolated by collagenase digestion (13). Following the isolation, the islets were incubated for 12-18 h in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) in the presence or absence of 15-50 U/ml recombinant mouse IL-1β (Sigma, St. Louis, MO). Nicotinamide (Wako Pure Chemical Industries Ltd., Osaka, Japan), 3AB (gifts from Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), 1,5-dihydroxyisoquinoline (1,5-DHIQ) (supplied by Kissei Pharmaceutical Co., Ltd., Matsumoto, Japan) or N^G-monomethyl-L-arginine (NMMA) monoacetate (Calbiochem-Novabiochem, La Jolla, CA) was added into the incubation medium 1 h before IL-1β.

Measurements of secreted insulin and nitrite: After the 18 h-culture of 20-30 islets, the islets were incubated in Krebs-Ringer bicarbonate buffer containing 0.2% bovine serum albumin (13) and 2.7 mM glucose for 30 min under an atmosphere of 95% O_2 and 5% CO_2 at 37°C. The islets were then incubated for a further 30 min in the buffer containing 20 mM glucose. Determinations of secreted insulin in the buffer were made by an insulin radioimmunoassay kit (Amersham) and rat insulin standard. Nitrite was analyzed as described (14).

Polymerase chain reaction (PCR) of reverse-transcripted mRNA: Total RNA (100 ng) extracted from the islets as described (15) was reverse-transcripted into cDNA at 42°C for 1 h in 20 μl of reverse transcriptase buffer (50 mM Tris-HCl (pH. 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT) containing 200 U of Superscript, 0.5 mM of dNTP, 1.1 U/μl of RNase inhibitor and 1.5 ng/μl of oligo$(dT)_{12\text{-}18}$. The reverse-transcripted sample (1 μl) was used for PCR amplifications. The sequences of the primers: for iNOS cDNA amplification were 5'-CGTGTGCCTGCTGCCTTCCTGCTGT-3' and 5'-GTAATCCTCAACCTGCTCCTCACTC-3' (nucleotides 2679 to 2703 and 3326 to 3350 in Ref. 16); for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA amplification were 5'-CATTGACCTCAACTACATGGT-3' and 5'-TTGTCATACCAGGAAATGAGC-3' (nucleotides 135 to 155 and 951 to 971 in Ref. 17); and for IRF-1 cDNA amplification were 5'-CTTCAGAGCTTAGGAGGCAGGGTCT-3' and 5'-AGCAGGCACAGGGCAAGGCACTATA-3' (nucleotides 1356 to 1380 and 1855 to 1879 in Ref. 18), respectively. PCR was performed in a thermal cycler (Perkin-Elmer, PJ1000) for 30 cycles for iNOS and GAPDH; for 28 cycles for IRF-1 with the following parameters: denaturation at 94°C for 30 sec, annealing at 62°C for 1 min, and extension at 72°C for 2 min. The PCR products were electrophoresed in 1.0% SeaKem GTG agarose containing ethidium bromide.

Measurement of ATP concentrations in the islets: Thirty islets were sonicated in 200 μl of ice-cold 8% (vol/vol) perchloric acid containing 2 mM EDTA and immediately plunged into dry ice-cold ethanol. The sonicated samples were neutralized with 1.5 N NaOH and diluted 100 times with 0.1 M Tris-acetate buffer (pH. 7.75) containing 2 mM EDTA. The ATP concentrations of the samples were measured by bioluminescence assay using an ATP monitoring kit (Bio Orbit, Turku, Finland). The fluorescence intensities were measured in a 1251 luminometer (Bio Orbit).

RESULTS AND DISCUSSION

As shown in Fig. 1, the IL-1β-stimulation of isolated pancreatic islets resulted in the inhibition of glucose-induced insulin secretion. Low-concentration nicotinamide (5 mM) partially, and high-concentration nicotinamide (20 mM) almost completely, attenuated the inhibition of insulin

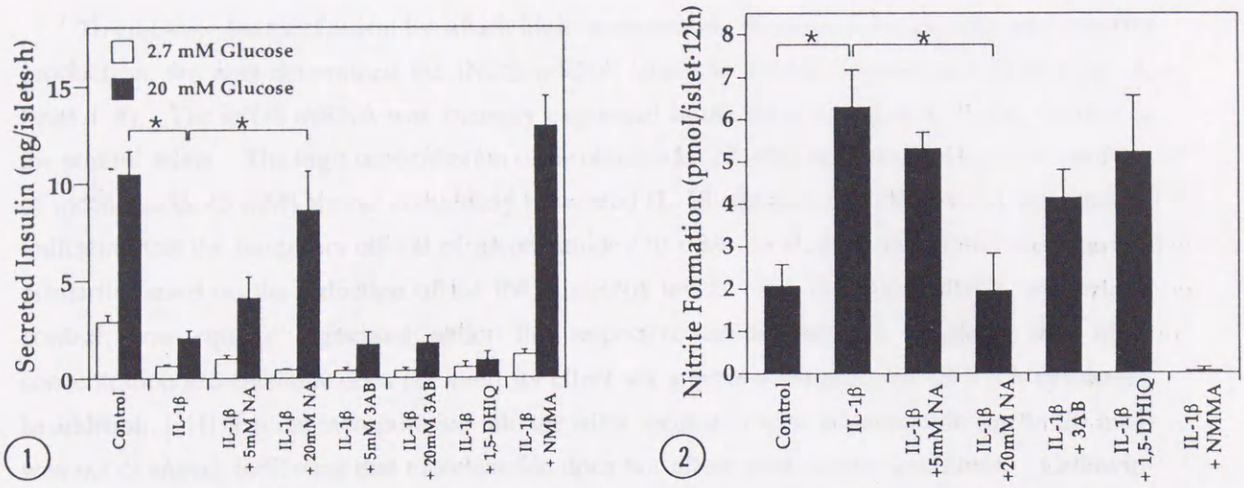


Fig. 1. Effects of poly(ADP-ribosyl)ation inhibitors and NMMA on the IL-1β-induced inhibition of insulin secretion. After 18-h incubation of isolated islets in the medium alone (control) or the medium containing 50 U/ml IL-1β, IL-1β + 5 mM NA (nicotinamide), IL-1β + 20 mM NA, IL-1β + 5 mM 3AB, IL-1β + 20 mM 3AB, IL-1β + 100 μM 1,5-DHIQ or IL-1β + 2 mM NMMA, insulin secretion under 2.7 mM glucose or 20 mM glucose was determined as in MATERIALS AND METHODS. N≥4 for each condition. *, $P < 0.01$. Vertical bars indicate S.E. Statistical analyses were performed by student's *t* test.

Fig. 2. Effects of poly(ADP-ribosyl)ation inhibitors and NMMA on nitrite formation from IL-1β-treated islets. Isolated islets were incubated at 37°C in the medium alone (control) or the medium containing 15 U/ml IL-1β, IL-1β + 5 mM NA (nicotinamide), IL-1β + 20 mM NA, IL-1β + 20 mM 3AB, IL-1β + 100 μM 1,5-DHIQ or IL-1β + 2 mM NMMA. After 12-h incubation, nitrite formation in the medium was determined as in MATERIALS AND METHODS. N≥4 for each condition. *, $P<0.01$. Vertical bars indicate S.E. Statistical analyses were performed using student's *t* test.

secretion at high glucose. Similar effects of high-concentration nicotinamide have been reported (11, 19, 20). However, 3AB (5 and 20 mM) and 1,5-DHIQ (100 μM), which completely inhibit poly(ADP-ribosyl)ation at these concentrations (21), failed to attenuate the inhibition of insulin secretion. This suggests that the attenuation by high-concentration nicotinamide can not be simply explained by the inhibition of poly(ADP-ribosyl)ation. Since NMMA, a NO synthase inhibitor, completely attenuated the inhibition of insulin secretion (Fig. 1), the inhibitory effect of IL-1β is mediated by NO. NO has been also reported to mediate cytokine-induced inhibition of insulin secretion in human islets (22). Although Rabinovitch *et al.* reported that human islet β cell destruction by cytokines was independent of NO production (23), they incubated human single β cells for a long time (84 h) in combination of cytokines.

We next incubated islets for 12 h with IL-1β in the presence or absence of various poly(ADP-ribose) synthetase inhibitors or NMMA. The nitrite levels in the medium of IL-1β-treated islets were about 3-fold higher than the control levels (Fig. 2), indicating that IL-1β induces NO production in pancreatic islets. NMMA (2 mM) completely inhibited IL-1β-induced nitrite formation, indicating that this nitrite accumulation was indeed derived from NO synthase. High-concentration nicotinamide (20 mM), but not low-concentration nicotinamide (5 mM), completely inhibited IL-1β-induced nitrite formation. However, 3AB (20 mM) and 1,5-DHIQ (100 μM) failed to inhibit nitrite formation.

To elucidate the mechanism by which high-concentration nicotinamide (20 mM) prevents NO production, we next determined the iNOS mRNA levels by reverse-transcripted PCR (Fig. 3, lanes 1-4). The iNOS mRNA was strongly expressed in the islets exposed to IL-1β, but not in the control islets. The high concentration of nicotinamide (20 mM) but not the low concentration of nicotinamide (5 mM) almost completely prevented IL-1β-stimulated iNOS mRNA expression, indicating that the inhibitory effects of nicotinamide (20 mM) on IL-1β-induced NO formation is primarily based on the reduction of the iNOS mRNA level. The GAPDH mRNA, an internal control, was equally expressed under the respective conditions; this suggests that high-concentration nicotinamide does not exert its effect via a general suppression of RNA synthesis. In addition, [^{3}H]-leucine incorporation into the islets incubated with nicotinamide (up to 20 mM) was not changed, indicating that nicotinamide does not affect total protein synthesis. Cetkovic-Cvrlje *et al.* reported that nicotinamide (10 and 20 mM) inhibits IL-1-induced NO production in RINm5F cells without decreasing iNOS mRNA expression (24), but RINm5F cells, a line of tumoral islet cells, are known to differ from normal islet cells in many biochemical and functional respects (25).

The promoter sequence of the murine iNOS gene (26) contains cytokine-responsive elements for the binding of transcriptional factors such as nuclear factor κB (NFκB) and IRF-1 (27). It has been suggested that NFκB activation is required for IL-1β-induced iNOS mRNA expression (28, 29). However, the recent study using IRF-1 knock-out mice indicated that IRF-1 is absolutely essential for iNOS mRNA induction (12). The IRF-1 mRNA is immediately induced when the cells are activated by various stimuli such as cytokines (30). We therefore determined the IRF-1 mRNA level (at 1 h after the exposure to IL-1β) and found that a high concentration of nicotinamide (20 mM) but not a low concentration of nicotinamide (5 mM) completely inhibits IL-1β-induced IRF-1 mRNA expression (Fig. 3, lanes 5-7). Thus, the inhibition of iNOS mRNA induction by high-concentration nicotinamide was thought to be achieved through the inhibition of IRF-1 mRNA induction.

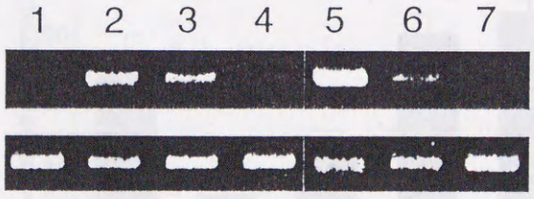


Fig. 3. Effects of nicotinamide on the expressions of iNOS mRNA and IRF-1 mRNA in IL-1β-treated islets. Isolated islets were incubated at 37°C in the medium alone (RPMI-1640 + 10 % FCS) (lane 1) or the medium containing 15 U/ml IL-1β (lanes 2 and 5), IL-1β + 5 mM nicotinamide (lanes 3 and 6), or IL-1β + 20 mM nicotinamide (lanes 4 and 7). After 12-h incubation (lanes 1-4), the iNOS mRNA and GAPDH mRNA (internal control) expressions were analyzed by reverse-transcripted PCR as in MATERIALS AND METHODS. Upper panel and lower panel (lanes 1-4) show the reverse-transcripted PCR products for iNOS mRNA and GAPDH mRNA, respectively. After 1-h incubation (lanes 5-7), the IRF-1 mRNA and GAPDH mRNA expressions were analyzed as in MATERIALS AND METHODS. Upper panel and lower panel (lanes 5-7) show the reverse-transcripted PCR products for IRF-1 mRNA and GAPDH mRNA, respectively. Two independent experiments gave similar results.

It has been suggested that NO inhibits iron-containing enzymes such as aconitase and complexes I and II by destroying their iron-sulfur centers (31). Since aconitase and complexes I and II, which are involved in Krebs cycle and electron transport respectively, play important roles for ATP generation in mitochondria, the ATP reduction in the IL-1β-treated islets was to be expected. As shown in Fig. 4, the IL-1β treatment reduced the intracellular ATP concentration to about 50 % of the control level. In contrast, low-concentration nicotinamide (5 mM) partially, and high-concentration nicotinamide (20 mM) completely, restored the ATP level. NMMA also completely restored the ATP level, indicating that IL-1β-evoked ATP depletion is indeed mediated by NO.

We have suggested that cyclic ADP-ribose plays a second messenger role in the glucose-induced insulin secretion by mobilizing Ca^{2+} from intracellular Ca^{2+} stores (32). In fact, intracellular Ca^{2+} elevation by glucose stimulation in the absence of extracellular Ca^{2+} has been recently confirmed in pancreatic β cells (33). We have proposed that ATP, generated in the course of glucose metabolism, plays a critical role in the glucose-induced insulin secretion by increasing cyclic ADP-ribose (34). ATP also regulates closure/opening of ATP-sensitive K^+ channels, which play a critical role in cell membrane depolarization, resulting in Ca^{2+}-influx (35). Therefore, the recovery of ATP concentrations by high-concentration nicotinamide may explain why this agent restores glucose-induced insulin secretion (Fig. 1).

From the results of the present study, the mechanisms by which nicotinamide prevents IL-1β-induced β cell dysfunction are as follows: (i) low-concentration nicotinamide (5 mM) prevents the IL-1β-evoked islet cell death by restoring cellular NAD^+ via the inhibition of poly(ADP-ribose) synthetase activation, but can not restore β cell functions such as insulin secretion, because cellular ATP is reduced by NO; (ii) high-concentration nicotinamide (20 mM) restores β cell functions by maintaining both NAD^+ and ATP levels because it prevents not only poly(ADP-ribose) synthetase activation but also iNOS gene expression by inhibiting IRF-1 mRNA induction.

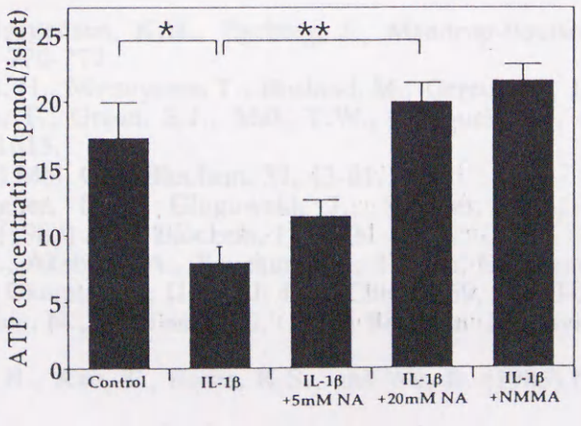


Fig. 4. Effects of nicotinamide and NMMA on IL-1β-induced reduction of intracellular ATP concentrations. Isolated islets were incubated in the medium alone (control) or the medium containing 50 U/ml IL-1β, IL-1β + 5 mM NA(nicotinamide), IL-1β + 20 mM NA or IL-1β + 2 mM NMMA. Following 18-h incubation, islets were incubated for 30 min in Krebs-Ringer bicarbonate buffer containing 20 mM glucose and then intracellular ATP concentrations were determined as in MATERIALS AND METHODS. N≥4 for each condition. *, $P < 0.02$; **, $P < 0.01$. Vertical bars indicate S.E. Statistical analyses were performed by student's *t* test.

Although administration of a NO synthase inhibitor such as NMMA *in vivo* decreases NO generation, NMMA causes hypertension by increasing peripheral vascular resistance (36). In contrast, no serious toxic effects of nicotinamide have been reported in human. Some clinical studies in diabetic patients showed that oral administration of nicotinamide protects residual β cell function including insulin secretion (37), and prevents Type I diabetes (38, 39). Large doses of nicotinamide have been also reported to have preventive effect on diabetes in nonobese diabetic mice, a model of Type I diabetes (40). The present results establish the molecular basis of the preventive effect of high-dose nicotinamide on Type I diabetes and emphasize the importance of the dose of nicotinamide used in the treatment of Type I diabetic patients.

ACKNOWLEDGMENTS: We thank Dr. Yoshikazu Kurashina of Kissei Pharmaceutical Co., Ltd. for gifts of 1,5-DHIQ and Brent Bell for critical reading of the manuscript. This work has been supported in part by Grants-in-Aid Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

1. Yamamoto, H., Uchigata, Y., and Okamoto, H. (1981) Nature 294, 284-286.
2. Uchigata, Y., Yamamoto, H., Kawamura, A., and Okamoto, H. (1982) J. Biol. Chem. 257, 6084-6088.
3. Okamoto, H. (1990) In Molecular Biology of the Islets of Langerhans. (H.Okamoto, Ed.) pp.209-231. Cambridge University Press, Cambridge, United Kingdom.
4. Heller, B., Wang, Z.-Q., Wagner, E.F., Radons, J., Bürkle, A., Fehsel, K., Burkart, V., and Kolb, H. (1995) J. Biol. Chem. 270, 11176-11180.
5. Sandler, S., Andersson, A., and Hellerström, C. (1987) Endocrinology 121, 1424-1431.
6. Corbett, J.A., Lancaster Jr, J.R., Sweetland, M.A., and McDaniel, M.L. (1991) J. Biol. Chem. 266, 21351-21354.
7. Green, I.C., Cunningham, J.M., Delaney, C.A., Elphick, M.R., Mabley, J.G., and Green, M.H.L. (1994) Biochem. Soc. Trans. 22, 30-37.
8. Delaney, C.A., Green, M.H.L., Lowe, J.E., and Green, I.C. (1993) FEBS Lett. 333, 291-295.
9. Bolaffi, J.L., Rodd, G.G., Wang, J., and Grodsky, G.M. (1994) Endocrinology 134, 537-542.
10. Ankarcrona, M., Dypbukt, J.M., Brüne, B., and Nicotera, P. (1994) Exp. Cell Res. 213, 172-177.
11. Andersen, H.U., Jørgensen, K.H., Egeberg, J., Mandrup-Poulsen, T., and Nerup, J. (1994) Diabetes 43, 770-777.
12. Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Koh, S.I., Kimura, T., Green, S.J., Mak, T.W., Taniguchi, T., and Vilček, J. (1994) Science 263, 1612-1615.
13. Okamoto, H. (1981) Mol. Cell. Biochem. 37, 43-61.
14. Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., and Tannenbaum, S.R. (1982) Anal. Biochem. 126, 131-138.
15. Kato, I., Suzuki, Y., Akabane, A., Yonekura, H., Tanaka, O., Kondo, H., Takasawa, S., Yoshimoto, T., and Okamoto, H. (1994) J. Biol. Chem. 269, 21223-21228.
16. Nunokawa, Y., Ishida, N., and Tanaka, S. (1993) Biochem. Biophys. Res. Commun. 191, 89-94.
17. Tso, J.Y., Sun, X.-H., Kao, T., Reece, K.S., and Wu, R. (1985) Nucleic Acids Res. 13, 2485-2502.
18. Yu-Lee, L.-Y., Hrachovy, J.A., Stevens, A.M., and Schwarz, L.A. (1990) Mol. Cell. Biol. 10, 3087-3094.
19. Buscema, M., Vinci, C., Gatta, C., Rabuazzo, M.A., Vignen, R., and Purrello, F. (1992) Metabolism 41, 296-300.
20. Eizirik, D.L., Sandler, S., Welsh, N., Bendtzen, K., and Hellerström, C. (1994) Autoimmunity 19, 193-198.

21. Banasik, M., Komura, H., Shimoyama, M., and Ueda, K. (1992) J. Biol. Chem. 267, 1569-1575.
22. Corbett, J.A., Sweetland, M.A., Wang, J.L., Lancaster Jr, J.R., and McDaniel, M.L. (1993) Proc. Natl. Acad. Sci. USA 90, 1731-1735.
23. Rabinovitch, A., Suarez-Pinzon, W.L., Strynadka, K., Schulz, R., Lakey, J.R.T., Warnock, G.L., and Rajotte, R.V. (1994) J. Clin. Endocr. Metab. 79, 1058-1062.
24. Cetkovic-Cvrlje, M., Sandler, S., and Eizirik, D.L. (1993) Endocrinology 133, 1739-1743.
25. Malaisse, W.J. (1990) In Molecular Biology of the Islets of Langerhans. (H.Okamoto, Ed.) pp.315-339. Cambridge University Press, Cambridge, United Kingdom.
26. Xie, Q.-W., Whisnant, R., and Nathan, C. (1993) J. Exp. Med. 177, 1779-1784.
27. Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T., and Taniguchi, T. (1988) Cell 54, 903-913.
28. Saldeen, J., and Welsh, N. (1994) Biochem. Biophys. Res. Commun. 203, 149-155.
29. Bedoya, F.J., Flodström, M., and Eizirik, D.L. (1995) Biochem. Biophys. Res. Commun. 210, 816-822.
30. Fujita, T., Reis, L.F.L., Watanabe, N., Kimura, Y., Taniguchi, T., and Vilček, J. (1989) Proc. Natl. Acad. Sci. USA 86, 9936-9940.
31. Corbett, J.A., Wang, J.L., Sweetland, M.A., Lancaster Jr, J.R., and McDaniel, M.L. (1992) J. Clin. Invest. 90, 2384-2391.
32. Takasawa, S., Nata, K., Yonekura, H., and Okamoto, H. (1993) Science 259, 370-373.
33. Rojas, E., Carroll, P.B., Ricordi, C., Boschero, A.C., Stojilkovic, S.S., and Atwater, I. (1994) Endocrinology 134, 1771-1781.
34. Takasawa, S., Tohgo, A., Noguchi, N., Koguma, T., Nata, K., Sugimoto, T., Yonekura, H., and Okamoto, H. (1993) J. Biol. Chem. 268, 26052-26054.
35. Ashcroft, F.M. (1988) Annu. Rev. Neurosci. 11, 97-118.
36. Haynes, W.G., Noon, J.P., Walker, B.R., and Webb, D.J. (1993) J. Hypertension 11, 1375-1380.
37. Vague, P., Picq, R., Bernal, M., Lassmann-Vague, V., and Vialettes, B. (1989) Diabetologia 32, 316-321.
38. Elliott, R.B., and Chase, H.P. (1991) Diabetologia 34, 362-365.
39. Pozzilli, P., Visalli, N., Signore, A., Baroni, M.G., Buzzetti, R., Cavallo, M.G., Boccuni, M.L., Fava, D., Gragnoli, C., Andreani, D., Lucentini, L., Matteoli, M.C., Crinò, A., Cicconetti, C.A., Teodonio, C., Paci, F., Amoretti, R., Pisano, L., Pennafina, M.G., Santopadre, G., Marozzi, G., Multari, G., Suppa, M.A., Campea, L., De Mattia, G.C., Cassone Faldetta, M., Marietti, G., Perrone, F., Greco, A.V., and Ghirlanda, G. (1995) Diabetologia 38, 848-852.
40. Yamada, K., Nonaka, K., Hanafusa, T., Miyazaki, A., Toyoshima, H., and Tarui, S. (1982) Diabetes 31, 749-753.

参考論文

1.Transgenic Mice Overexpressing Human Vasoactive Intestinal Peptide (VIP) Gene in Pancreatic β Cells; Evidence for Improved Glucose Tolerance and Enhanced Insulin Secretion by VIP and PHM-27 *in vivo*.
Ichiro Kato, Yu Suzuki, Atsuya Akabane, Hideto Yonekura, Osamu Tanaka, Hisatake Kondo, Shin Takasawa, Takashi Yoshimoto and Hiroshi Okamoto
The Journal of Biological Chemistry, 269, 21223-21228, 1994

2.Regulatory Role of CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase) in Insulin Secretion by Glucose; Enhanced Insulin Secretion in CD38 Transgenic Mice.
Ichiro Kato, Shin Takasawa, Atsuya Akabane, Osamu Tanaka, Hiroshi Abe, Toshinari Takamura, Yu Suzuki, Koji Nata, Hideto Yonekura, Takashi Yoshimoto and Hiroshi Okamoto.
The Journal of Biological Chemistry, *in press*

3.膵ランゲルハンス島β細胞でヒトVIP遺伝子を過剰発現するトランスジェニックマウスの作製と導入遺伝子による血糖降下作用.
加藤一郎、鈴木　雄、赤羽敦也、田中　理、近藤尚武、米倉秀人、岡本　宏
分子糖尿病学, 5, 73-80, 1994

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 33, Issue of August 19, pp. 21223-21228, 1994
Printed in U.S.A.

Transgenic Mice Overexpressing Human Vasoactive Intestinal Peptide (VIP) Gene in Pancreatic β Cells

EVIDENCE FOR IMPROVED GLUCOSE TOLERANCE AND ENHANCED INSULIN SECRETION BY VIP AND PHM-27 *IN VIVO**

(Received for publication, January 28, 1994, and in revised form, April 26, 1994)

Ichiro Kato‡, Yu Suzuki‡, Atsuya Akabane‡, Hideto Yonekura‡, Osamu Tanaka§, Hisatake Kondo§, Shin Takasawa‡, Takashi Yoshimoto¶, and Hiroshi Okamoto‡‖

From the Departments of ‡Biochemistry, §Anatomy, and ¶Neurosurgery, Tohoku University School of Medicine, Sendai 980-77, Miyagi, Japan

Vasoactive intestinal peptide (VIP), a 28-amino acid peptide hormone, plays many physiological roles in the peripheral and central nervous systems. It has been proposed that endogenous VIP released from VIP-containing nerves is involved in the regulation of the secretory function of the endocrine pancreas. To test this hypothesis *in vivo*, we produced transgenic mice carrying the human VIP/peptide histidine methionine 27 (PHM-27) gene under the control of insulin promoter. In immunohistochemical analyses of islets, all the islet β cells of transgenic mice were intensely stained for both VIP and PHM-27, consistent with the fact that these two peptides are encoded in a single mRNA (Itoh, N., Obata, K., Yanaihara, N., and Okamoto, H. (1983) *Nature* 304, 547–549). VIP was efficiently secreted from isolated transgenic islets *in vitro*. The blood glucose assays in free-fed mice indicated that the transgene lowered the blood glucose levels of transgenic mice (128 ± 4 mg/dl) by about 20% below control levels (155 ± 6 mg/dl). In the glucose tolerance test, at 60 min after glucose administration, the transgenic blood glucose levels (129 ± 12 mg/dl) were much lower than control levels (175 ± 13 mg/dl). The transgenic serum insulin levels at 15 min after glucose administration were 2.5–3.0-fold higher than control levels. The transgene was also effective in ameliorating glucose intolerance of 70% depancreatized mice. These results indicate that VIP and PHM-27 produced from the transgenic β cells efficiently enhance glucose-induced insulin secretion from β cells by an autocrine mechanism. These results also suggest that genetic manipulation of islet β cells by the human VIP/PHM-27 gene or delivery of VIP to β cells may ultimately provide a valuable approach to enhancing insulin secretion in clinical diabetes.

Vasoactive intestinal peptide (VIP)[1] is a 28-amino acid C-terminally amidated peptide hormone that belongs to the family of brain-gut peptide hormones (1, 2). In human, VIP and peptide histidine methionine 27 (PHM-27) are encoded in a single mRNA and are structurally related to each other (3). VIP and PHM-27 are cleaved from a 170-amino acid precursor protein (3) and are finally amidated at the C terminus by peptidylglycine monooxygenase (4–6). VIP and VIP-immunoreactive substances are found in various tissues such as lung, brain, intestine, and pancreas (7–10) and seem to work as neurotransmitters or neuromodulators in the central and peripheral nervous systems. VIP induces relaxation of smooth muscle (11) and stimulates secretion of electrolytes from the gastrointestinal tract (12). VIP also stimulates glycogenolysis in the cerebral cortex (13) and regulates cerebral blood flow (14).

The pancreatic islets receive substantial innervation by sympathetic, sensory, and parasympathetic neurons (8). VIP-containing nerves have been shown to be immunolocalized around and within pancreatic islets in various mammals and humans (7, 10). VIP has been shown to stimulate insulin secretion from perfused pancreas (15). In addition, the existence of specific VIP receptors in pancreatic β cells was demonstrated by quantitative electron microscopic autoradiography (16). These findings suggest the importance of VIP as a neurotransmitter or neuromodulator of pancreatic islets. However, when VIP was injected intravenously in mouse (17), in rats (18), and in human (10), the blood glucose level rose or remained unchanged. Until now, there has been no evidence that VIP possesses the ability to reduce blood glucose *in vivo* by enhancing insulin secretion from pancreatic islets.

Previous studies have relied on the systemic administration of VIP to investigate its biological role *in vivo*. Recent technical advances in genetic manipulation of mouse embryo have enabled us to directly target the expression of proteins in specific cells. To determine how VIP controls the function of pancreatic islets, we have produced and examined transgenic mice that overexpress human VIP gene in pancreatic β cells. VIP and PHM-27 were efficiently synthesized and secreted from β cells of transgenic mice, resulting in the enhancement of glucose-induced insulin secretion and the reduction of blood glucose *in vivo*. The transgene also significantly ameliorated glucose intolerance of 70% depancreatized mice. These results indicate that VIP and PHM-27 play important roles in regulating the secretory function of pancreatic islets and suggest the possible therapeutic significance of these peptides in clinical diabetes.

EXPERIMENTAL PROCEDURES

Construction of Rat Insulin II Promoter/Human VIP Hybrid Gene—We have previously isolated and determined the human VIP/PHM-27 genomic sequence (19). Human (3) and mouse (20) VIP have identical amino acid sequences. The rat insulin II promoter, previously reported to be active in pancreatic β cells of transgenic mice (21), was used to direct expression to pancreatic β cells. The *Xma*I sites were

* This work has been supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom all correspondence should be addressed: Dept. of Biochemistry, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, 980-77, Miyagi, Japan. Tel.: 81-22-273-9061; Fax: 81-22-272-8101.

[1] The abbreviations used are: VIP, vasoactive intestinal peptide; PHM-27, peptide histidine methionine 27; kbp, kilobase pair(s); RIA, radioimmunoassay.

introduced into the rat insulin II gene exon I at position +22 (22) and into the human VIP gene exon I at position +25 from the transcription initiation sites using polymerase chain reaction. The 0.7-kbp *Bam*HI-*Xma*I fragment of the rat insulin II promoter (nucleotides −695 to +22 in Ref. 22) and the 9.8-kbp *Xma*I-*Pst*I fragment of the entire human VIP gene were ligated at the *Xma*I site in the correct orientation (see Fig. 1). The rat insulin II promoter/human VIP hybrid gene (10.5 kbp) was separated from the plasmid by *Bam*HI and designated Ins-VIP. Ins-VIP DNA was purified by agarose gel electrophoresis and by NACS prepac cartridge (Life Technologies, Inc.). The DNA dissolved in 10 mM Tris, 0.1 mM EDTA (pH 7.6) at a concentration of 2 μg/ml was microinjected.

Production and Identification of Transgenic Mice—The production of transgenic mice was performed according to standard procedures (23). In brief, a DNA solution (2 μg/ml) was microinjected into male pronuclei of fertilized mouse eggs taken from superovulated ICR or F_1 (C57Bl/6J × CBA/J) females. The injected eggs were surgically transferred to the oviducts of ICR pseudopregnant female mice. Identification of transgenic mice was performed by polymerase chain reaction on genomic DNA isolated from mouse tail (23).

Southern and Northern Blot Analyses—Southern and Northern blot analyses were carried out as described (24) using a ^{32}P-labeled human VIP cDNA probe (*Sph*I-*Eco*RI fragment of 312 base pairs; nucleotides 244–556 in Ref.3). Hybridization signals were scanned with a bioimage analyzer, BAS 2000 (Fuji Photo Co., Ltd., Tokyo, Japan) and compared with those of a human control sample.

Immunohistochemical Analyses—Approximately one-third of the pancreas adjacent to the spleen was used for histology. The tissue was fixed in 4% paraformaldehyde, 0.1 M sodium phosphate buffer (pH 7.3) overnight at 4 °C and placed in 30% sucrose. After embedding, 10-μm sections were cut and collected onto polylysine-coated slides. The sections were incubated with the antisera to VIP, PHM-27, or insulin followed by visualization with the avidin-biotin peroxidase method (Vector Laboratories) using diaminobenzidine as the chromogen. Primary antibodies were used at the following dilutions: VIP at 1:1500 (Amersham), PHM-27 at 1:1500 (Affinity Research Products), insulin at 1:500 (Dako).

Preparations of Mouse Islet Extract and Serum for Radioimmunoassay of VIP—Transgenic and nontransgenic islets were isolated in parallel from 6- to 10-week-old litters by the collagenase digestion method (25). Groups of 100 hand-picked islets were washed two times with the homogenizing buffer (50 mM sodium phosphate (pH 7.2) containing 0.4% bovine serum albumin and 10 mM EDTA), homogenized in the presence of 1 ml of homogenizing buffer containing 160 μg/ml aprotinin, sonicated at 4 °C for 10 s, and stored at −30 °C until radioimmunoassay (RIA) of VIP using the VIP RIA kit (Amersham) and human VIP standards. Blood samples sufficient for RIA were obtained by decapitation from free fed mice between 8:00 and 10:00 p.m. Serum samples were prepared by centrifugation after incubating blood samples overnight at 4 °C.

Measurement of Glucose, Insulin, and VIP—Blood glucose determinations were made on whole blood using the Accucheck II (Boehringer Manheim) between 8:00 and 10:00 p.m. Determinations of insulin levels in serum samples were made by RIA using the insulin RIA kit (Novo Nordisk Biolabs) and rat insulin standards. Determinations of VIP levels were made by RIA. All assays were performed in duplicate. All statistical analyses were performed by Student's *t* test.

Generation of 70% Depancreatized Mice—Transgenic and nontransgenic littermate male mice (age, 8 weeks) were anesthetized by pentobarbital and subjected to a partial pancreatectomy. The splenic and gastric portions of the pancreas were surgically removed, leaving the duodenal portion intact. This procedure allowed the removal of 70% of the whole pancreas in weight. Seven days after the partial pancreatectomy, 12-h fasted mice were subjected to glucose tolerance tests.

RESULTS

Generation of Transgenic Mice Carrying the Human Vasoactive Intestinal Peptide Gene Ligated to Rat Insulin II Promoter—The rat insulin II promoter/human VIP hybrid gene (Fig. 1) was designed to direct the overexpression of the human VIP gene in pancreatic β cells of transgenic mice. The hybrid gene contained the entire human VIP gene (19) ligated to the rat insulin II promoter (22). The gene fragment (Ins-VIP, see "Experimental Procedures") was injected into fertilized eggs of ICR or (C57Bl/6J X CBA/J) F_1 mice. Four out of 69 newborn mice were found to carry the Ins-VIP transgene, as detected by polymerase chain reaction analyses using primers for the insulin promoter and VIP gene (data not shown). Founder mice were mated with ICR mice to obtain littermates. The transgenic strain 330 (derived from ICR egg) and the strains 361 and 366 (derived from F_1 eggs) transmitted the transgene into their offspring and were further maintained on ICR mice background.

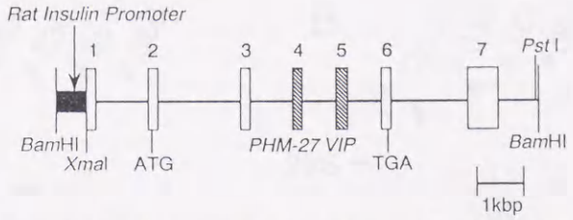


FIG. 1. **Schematic representation of the rat insulin II/human VIP fusion gene used for microinjection.** The rat insulin II/human VIP fusion gene consists of 0.7 kbp of the promoter and 22 base pairs of the 5′-noncoding region of rat insulin II gene (*solid box*) and the entire human VIP gene extending from 25 base pairs of the 5′-noncoding region of exon 1 up to 0.9 kbp of the 3′-flanking region. Locations of exons of the human VIP gene are indicated by *numbered boxes*; *thin lines* show introns and 3′-flanking sequence. Translation initiation (*ATG*) and termination (*TGA*) codons and relevant restriction sites used for the transgene construction are indicated. *PHM-27* and *VIP* are encoded in exon 4 and exon 5 (*striped boxes*), respectively.

Southern and Northern blot Analyses of the Transgenes—The copy number of the integrated Ins-VIP construct in the three strains was examined by Southern blot analysis using a human VIP cDNA probe. Correct bands were observed in every strain (Fig. 2*A*). The transgene copy numbers were estimated by comparison with the human endogenous gene: strain 330 (8 copies per haploid genome), strain 361 (26 copies), and strain 366 (4 copies).

Northern blot analysis using the human VIP cDNA probe showed that all three transgenic strains expressed human VIP mRNA in the pancreas (Fig. 2*B*). The human VIP mRNA expression in other tissues such as brain, lung, heart, intestine, kidney, and liver was not detected in the transgenic mice (data not shown).

Immunohistochemical Analyses of Pancreatic Islets—Cryostat sections of pancreas were examined after treatment with the polyclonal antibody that reacts to VIP, PHM-27, or insulin. The islets of the transgenic mouse were densely and almost entirely stained for both VIP (Fig. 3*D*) and PHM-27 (Fig. 3*E*), indicating that the transgenes exhibited tissue-specific expression and were correctly translated. These results are also consistent with the fact that VIP and PHM-27 are synthesized from a single mRNA (3). On the other hand, examination of pancreatic tissue from the nontransgenic mouse showed immunoreactivity for neither VIP nor PHM-27 in the islets (Fig. 3, *A* and *B*). In contrast to islets, the pancreatic exocrine cells showed no detectable staining for VIP nor PHM-27 in any of the transgenic and nontransgenic mice. VIP-positive nerve fibers and ganglions were seen close to the periphery of nontransgenic islets (Fig. 3*A*, *arrows*), suggesting a possible regulatory role of VIP in the function of islets. Both transgenic and nontransgenic islets were well stained for insulin (Fig. 3, *C* and *F*).

Radioimmunoassay of VIP in Islets and Serum—As shown in Table I, the VIP concentrations in the islets of the three transgenic mice were much higher than that in control mice. Serum VIP concentrations in transgenic mice were also higher than that of control mice. These results indicate that, in transgenic mice, VIP is released from pancreatic islets into whole blood circulation in the same way as insulin. The serum VIP levels in these transgenic mice were below the levels seen in patients with VIP-producing tumors (160–500 fmol/ml in Ref. 26). This may explain why the watery diarrhea peculiar to VIP-producing tumors was not seen in these transgenic mice.

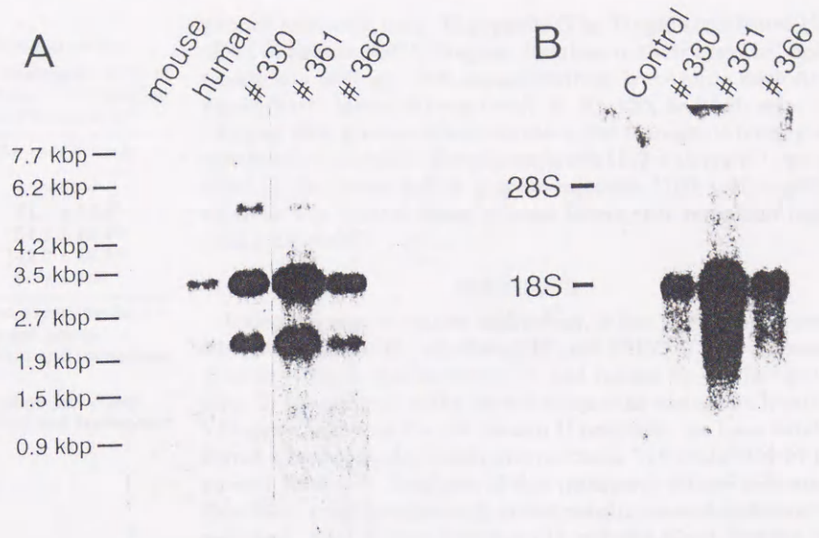


FIG. 2. **Southern blot analysis of genomic DNA (*A*) and Northern blot analysis of pancreas RNA (*B*) from transgenic mice strains 330, 361, 366 and control mice.** *A*, a human DNA sample was loaded as control. *Eco*RI-digested genome DNA (5 μg) was used for DNA blot hybridization. Positions of size markers are presented on the *left*. The 3.2- and 2.2-kbp *Eco*RI fragments were obtained in all transgenic mice as seen in human sample. The endogenous mouse VIP genome fragment did not hybridize under the conditions used in this study. *B*, total RNA (5 μg) isolated from mouse pancreas was used for RNA blot hybridization. Positions of mouse 28 S and 18 S rRNAs are presented on the *left*.

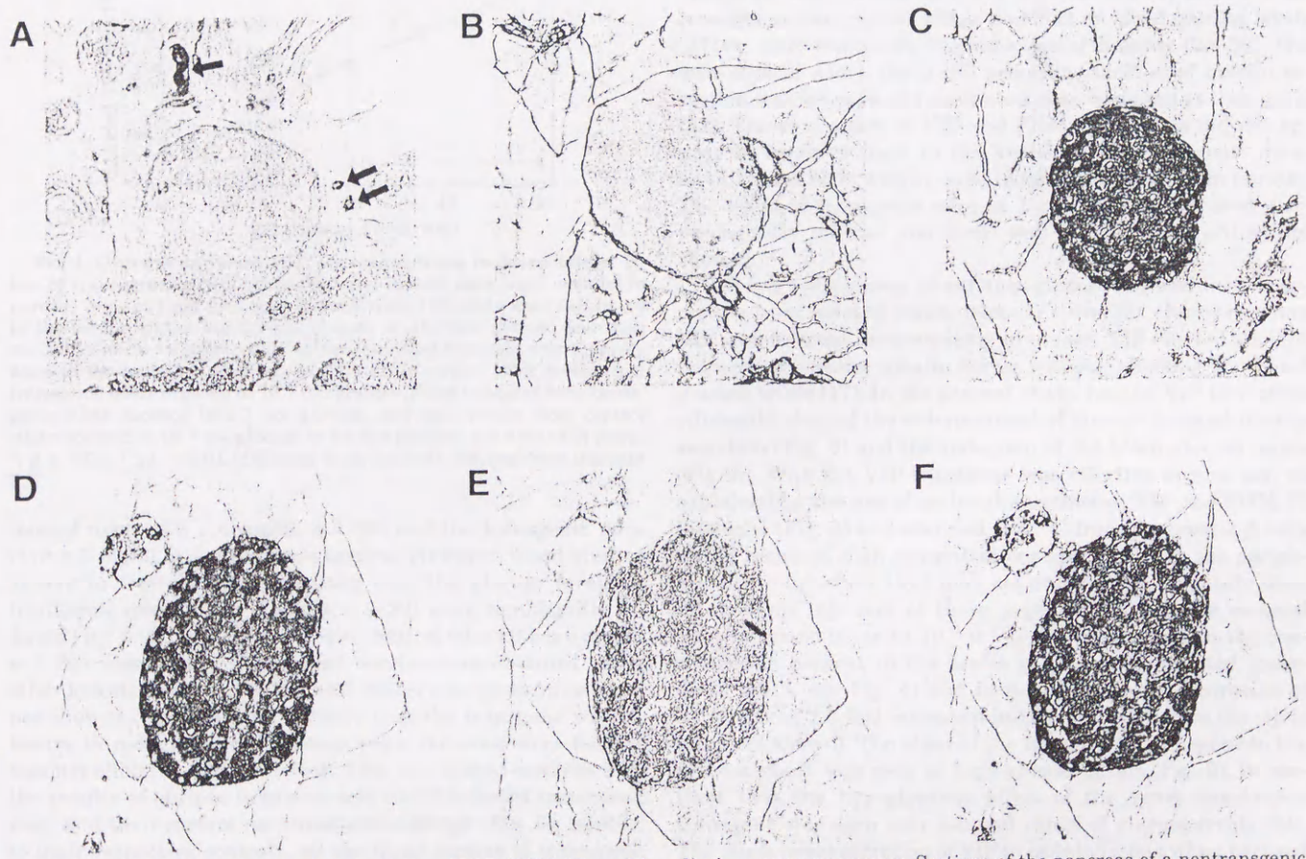


FIG. 3. **Immunohistochemical detections of VIP, PHM-27, and insulin in mouse pancreas.** Sections of the pancreas of a nontransgenic control mouse (*A, B, C*) and of a transgenic mouse strain 330 (*D, E, F*) were incubated with the antiserum against VIP (*A, D*), against PHM-27 (*B, E*), or against insulin (*C, F*) followed by visualization with the avidin-biotin peroxidase method. VIP-positive nerve fibers and ganglions close to the periphery of nontransgenic islets are indicated by *arrows* (*A*).

Glucose-induced VIP Secretion from Isolated Islets—Since islet β cells secrete insulin from β cell granules by the stimulation of glucose, glucose-induced VIP secretion from islets of transgenic mice is expected. We isolated pancreatic islets from transgenic and control mice and exposed them to media containing 2.7 mM glucose or 16.7 mM glucose. As shown in Fig. 4, when transgenic islets were exposed to media containing 2.7 mM glucose, VIP secretion was 12.5 ± 6.9 fmol/60 min/100 islets. When transgenic islets were exposed to media containing 16.7 mM glucose, VIP secretion was 100.2 ± 26.9 fmol/60 min/100 islets. These results demonstrate that VIP secretion from transgenic islets is greatly facilitated by high glucose (16.7 mM) concentration. In contrast, VIP secretion from nontransgenic islets was not at all detected under low glucose (2.7 mM) nor high glucose (16.7 mM) concentrations.

Blood Glucose Analyses—Blood glucose levels were determined on the strain 330 after 12 h fasting. There were no significant differences in the levels of blood glucose between the

TABLE I

Radioimmunoassay of VIP levels in mouse islets and serum

Islet extracts and serum samples prepared from transgenic mice of strains 330, 361, and 366 and control mice were subjected for radioimmunoassay of VIP as described under "Experimental Procedures."

Mice	Islet VIP levels	Serum VIP levels
	pmol/100 islets	*fmol/ml*
Control	0.029 ± 0.003[a]	21.1 ± 0.95[b]
Strain 330	1.97 ± 0.23[a,c]	54.2 ± 12.4[d,e]
Strain 361	5.88 ± 0.41[a,c]	142.0 ± 46.8[d,e]
Strain 366	2.17 ± 0.23[a,c]	58.0 ± 7.0[c,d]

[a] Values are the mean ± S.E. of three independent experiments.
[b] Values are the mean ± S.E. of four control mice per group.
[c] $p < 0.001$, indicating the difference between control and transgenic mice.
[d] Values are the mean ± S.E. of three transgenic mice per group.
[e] $p < 0.05$, indicating the difference between control and transgenic mice.

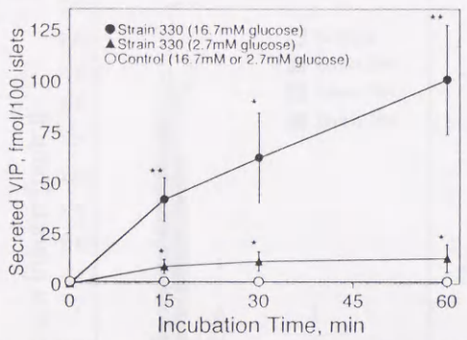


FIG. 4. **Glucose-induced VIP secretion from isolated islets.** Islets of transgenic mice of strain 330 and control mice were isolated in parallel from at least four mice of each type. 100 islets were incubated in the media containing 2.7 mM glucose or 16.7 mM glucose and then medium samples were removed at the indicated time and subsequently assayed for radioimmunoassay of VIP. *Filled circles* show levels from transgenic islets exposed to 16.7 mM glucose, *filled triangles* from transgenic islets exposed to 2.7 mM glucose, and *open circles* from control islets exposed to 16.7 mM glucose or 2.7 mM glucose. $n = 3$ for each point. *, $p < 0.05$; **, $p < 0.01$ (different from control). *Vertical bars* indicate S.E.

control mice (106 ± 4 mg/dl, $n = 29$) and the transgenic mice (110 ± 5 mg/dl, $n = 29$) during fasting. However, blood glucose assays in free-fed mice indicated that the glucose levels in transgenic mice (128 ± 4 mg/dl, $n = 29$) were significantly reduced ($p < 0.001$) as compared with control mice (155 ± 6 mg/dl, $n = 20$). Essentially the similar results were obtained using other two strains (strains 361 and 366) of transgenic mice (data not shown). These results indicate that the transgene was effective in reducing blood glucose when the mice were fed but was not effective during fasting. This was indeed confirmed by the results of glucose tolerance test on 12-h fasted transgenic mice and their control nontransgenic siblings (Fig. 5); relative to their respective controls, all the three strains of transgenic mice had significantly lower glucose levels at 30, 60, 90, and 120 min.

Serum Insulin Levels after Glucose Administration—Serum insulin levels at each point in the glucose tolerance test were measured. As shown in Fig. 6, at 15 min after glucose administration, the transgenic serum insulin levels were 2.5–3.0-fold higher than control levels. This result demonstrates that VIP and PHM-27 produced by the transgene promptly enhanced glucose-induced insulin secretion *in vivo*. This result also suggests that the altered whole animal glucose homeostasis is caused by a function of enhanced insulin secretion rather than a nutritional or peripheral metabolic effect of VIP and PHM-27.

Glucose Tolerance Tests in 70% Depancreatized Mice—A week after the 70% pancreatectomy, mice were subjected to glucose tolerance tests. The results (Fig. 7) again confirmed the effect of human VIP transgene. Relative to their operated nontransgenic siblings, 70%-depancreatized transgenic mice had significantly lower glucose levels at 90, 120, and 180 min. At 180 min after glucose administration, the transgenic blood glucose levels returned to the glucose levels (112 ± 18 mg/dl) equivalent to the levels before glucose injection (108 ± 10 mg/dl), whereas the control blood glucose levels still remained high (165 ± 15 mg/dl).

DISCUSSION

Using transgenic mouse technology, it has now become possible to test directly whether VIP and PHM-27 can enhance glucose-induced insulin secretion and reduce blood glucose *in vivo*. In the present study, by microinjecting the entire human VIP gene ligated to the rat insulin II promoter, we have established a mouse model which overproduces VIP and PHM-27 in pancreatic β cells. Analyses of this transgenic mouse indicated that the transgene efficiently enhances glucose-induced insulin secretion (Fig. 6) and significantly reduces blood glucose as compared with control mice (Fig. 5). Previous reports on β cell transgenes have shown either no effect on blood glucose levels (27) or, more commonly, the induction of diabetes (28–30). The only case in which the β cell transgene facilitated insulin secretion was when yeast hexokinase gene was used as transgene (31). The production of VIP and PHM-27 in islets did not appear to be deleterious to the health of the transgenic mice; fertility and body weight were indistinguishable from normal. The islets of transgenic mice at 1 year of age appeared morphologically normal and were well stained for insulin (not shown).

VIP has been shown to enhance glucose-induced insulin secretion from isolated mouse islets *in vitro* (32). However, when VIP was injected intravenously in mouse, VIP elicited a slight increase of plasma insulin levels without affecting the blood glucose levels (17). In the present study, human VIP transgene efficiently elicited the enhancement of glucose-induced insulin secretion (Fig. 6) and the reduction of the blood glucose levels (Fig. 5). Why the VIP transgene was effective *in vivo* can be explained by the use of an insulin promoter; VIP and PHM-27 produced (Fig. 3) and secreted (Fig. 4) from pancreatic β cells would result in high concentrations specifically in the periphery of β cells where they may act much more efficiently than intravenous injection of these peptides. In fact, we exposed nontransgenic islets to 10^{-9} M VIP (corresponding to the concentration present in the media of glucose-stimulated transgenic islets, see Fig. 4) and found that this concentration of VIP-induced 2.0-fold increased insulin secretion from the islets (data not shown). The effect of the human VIP transgene in the present study was seen at high glucose levels (Fig. 5), in contrast that the hypoglycemic effect of the yeast hexokinase transgene was seen over a broad range of glucose levels (31). The much lower secretion of VIP in isolated islets when exposed to media containing low glucose concentration (Fig. 4) and the less stimulatory effect of VIP on insulin release at low glucose levels (32) may explain why the hypoglycemic effect of the human VIP transgene is not observed at low glucose levels.

Although VIP is not endogenously produced in pancreatic islets of control mice (Fig. 3*A*), VIP-containing nerves are localized around and/or within islets in mice (Fig. 3*A*, *arrows*), in rats (7), and in humans (7, 10). VIP is found in neurons containing acetylcholine (33), and the signal induced by acetylcholine is shown to be modulated by VIP (34). In addition, VIP is released from intrapancreatic nerve fibers in the pig after electrical stimulation of the vagus nerve (35). These observations, together with the results of the present study using transgenic mice, suggest that endogenous VIP, which would be released

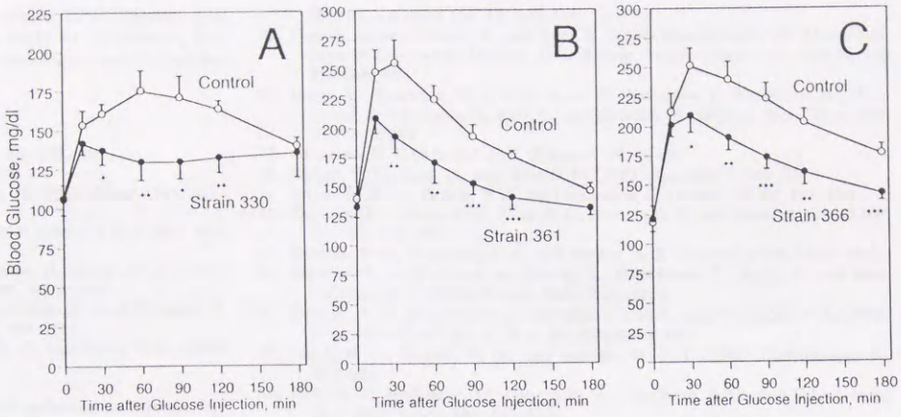


FIG. 5. **Glucose tolerance test.** Transgenic mice of strains 330 (*A*), 361 (*B*), and 366 (*C*) and their nontransgenic siblings (*Control*) were fasted 12 h and then administered an intraperitoneal injection of 2 mg of glucose/g (body weight). Blood samples were taken at the indicated times from the tail vein. *Filled circles* show levels from transgenic mice, *open circles* from control mice. $n = 8$ (strains 330, 361, and 366) and 8 (*Control*). *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$ (different from control). *Vertical bars* indicate S.E.

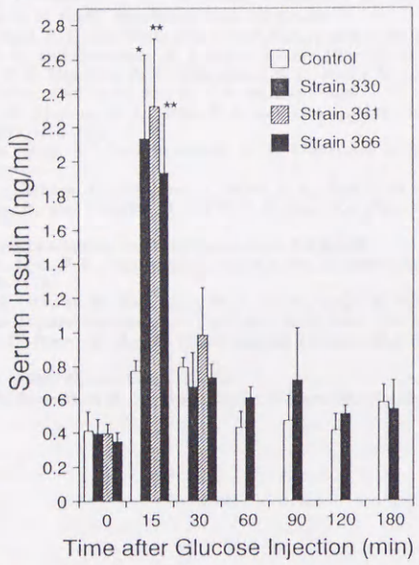


FIG. 6. **Serum insulin levels in glucose tolerance test.** Age-matched transgenic mice of strains 330, 361, and 366 and nontransgenic mice (*Control*) were fasted 12 h and then administered an intraperitoneal injection of 2 mg of glucose/g (body weight). Mice were sacrificed to obtain samples for measurement of serum insulin levels at each point after glucose administration. Determinations of serum insulin levels were made by RIA. *Shaded, striped,* and *solid bars* show levels from strains 330, 361, and 366, respectively, and *open bars* from control mice. At least six determinations were obtained for each point. *, $p < 0.01$; **, $p < 0.001$ (different from control). *Vertical bars* indicate S.E.

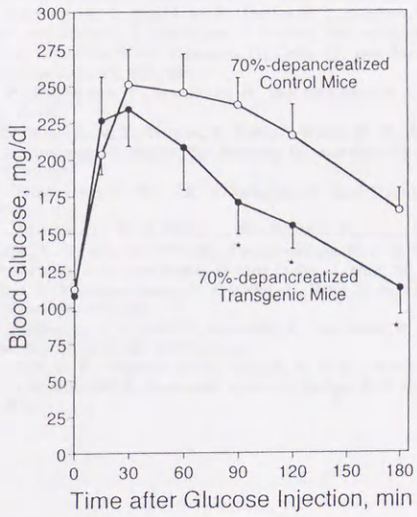


FIG. 7. **Glucose tolerance test in the 70% depancreatized mice.** Seven days after 70% pancreatectomy, 12-h-fasted mice were subjected to glucose tolerance tests as in the legend to Fig. 5. *Filled circles* show levels from transgenic mice of the strain 330, *open circles* from control mice. $n = 7$ (transgenic mice, strain 330) and 7 (control mice). *, $p < 0.05$ (different from control). *Vertical bars* indicate S.E.

from nerve terminals in response to various stimulations, such as a meal, potentiates glucose-induced insulin secretion *in vivo*. The stimulation of insulin release by VIP may also explain the marked elevation of serum insulin that occurs during a meal despite the rather modest increase in the blood glucose level (36).

In human, VIP and PHM-27 are encoded in a single mRNA and their structures are closely related (3). We have previously shown that peptide histidine isoleucine, a rat (37) and mouse (20) homologue to human PHM-27, also enhances glucose-induced insulin secretion *in vitro* (38). PHM-27 as well as VIP efficiently bound to the cloned rat VIP receptor expressed on the cell surface of COP cells and accumulated intracellular cAMP (39). It is thus possible that PHM-27 as well as VIP secreted from transgenic islets efficiently bind to β cell surface VIP receptors (16), facilitating insulin secretion by an autocrine mechanism.

VIP and PHM-27 produced from the transgene significantly ameliorated glucose intolerance even when 70% of pancreatic tissue was surgically removed (Fig. 7). This result demonstrates that the transgenic mice are resistant to reductions of functional islet cell mass. This is clinically significant, since the reduction of islet cell mass is often a main cause of glucose intolerance (40) in insulin-dependent diabetes mellitus. In non-insulin-dependent diabetes mellitus, glucose-induced insulin secretion is impaired (41) even when pancreatic islets retain significant amounts of insulin (42). The results of the present study also suggest that the human VIP transgene can enhance glucose-induced insulin secretion and thereby reduce blood glucose levels in non-insulin-dependent diabetes mellitus. The lack of apparent toxicity of VIP expression in islets provides encouragement for the feasibility of somatic cell gene therapy directed to diabetic islets. In addition, the limited effect of VIP transgene at relatively high blood glucose levels (Fig. 5) eliminates the danger of hypoglycemia, which sometimes occurs in the treatment of diabetic patients with insulin or sulfonylureas. Genetic manipulation of the β cell *in situ* is not presently feasible. However, by using plasmid DNA complexed with cationic liposomes (43), successful gene therapy of cystic fibrosis mice *in situ* has been reported recently (44). VIP gene transfer into islets *in situ*, although difficult, would provide a means to improve the secretory function of the diabetic islets. Another possibility is to deliver these peptides specifically to islets, thereby providing a valuable approach to enhancing insulin secretion in clinical diabetes.

Acknowledgments—We are grateful to Professor C. J. Epstein and E. Carlson (Department of Pediatrics, University of California, San Francisco) for teaching us the microinjection technique and B. Bell for reading the manuscript.

REFERENCES

1. Said, S. I., and Mutt, V. (1972) *Eur. J. Biochem.* **28,** 199–204
2. Said, S. I. (1986) *J. Endocrinol. Invest.* **9,** 191–200
3. Itoh, N., Obata, K., Yanaihara, N., and Okamoto, H. (1983) *Nature* **304,** 547–549
4. Noguchi, M., Takahashi, K., and Okamoto, H. (1988) *Tohoku J. Exp. Med.* **156,** 191–207
5. Tajima, M., Iida, T., Yoshida, S., Komatsu, K., Namba, R., Yanagi, M., Noguchi, M., and Okamoto, H. (1990) *J. Biol. Chem.* **265,** 9602–9605
6. Kato, I., Yonekura, H., Tajima, M., Yanagi, M., Yamamoto, H., and Okamoto, H. (1990) *Biochem. Biophys. Res. Commun.* **172,** 197–203
7. Bishop, A. E., Polak, J. M., Green, I. C., Bryant, M. G., and Bloom, S. R. (1980) *Diabetologia* **18,** 73–78
8. Miller, R. E. (1981) *Endocr. Rev.* **2,** 471–494
9. Said, S. I. (ed) (1982) *Vasoactive Intestinal Peptide: Advances in Peptide Hormone Research Series,* Raven Press, New York
10. Fahrenkrug, J., Pederson, J. H., Yamashita, Y., Ottesen, B., Hökfelt, T., and Lundberg, J. M. (1987) *Regulatory Pept.* **18,** 51–61
11. Piper, P. J., Said, S. I., and Vane, J. R. (1970) *Nature* **225,** 1144–1146
12. Barbezat, G. O., and Grossman, M. I. (1971) *Science* **174,** 422–424
13. Magistretti, P. J., Morrison, J. H., Shoemaker, W. J., Sapin, V., and Bloom, F. E. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 6535–6539
14. Heistad, D. D., Marcus, M. L., Said, S. I., and Gross, P. M. (1980) *Am. J. Physiol.* **239,** H73–H80
15. Schebalin, M., Said, S. I., and Makhlouf, G. M. (1977) *Am. J. Physiol.* **232,** E197–E200
16. Anteunis, A., Hejblum, G., Astesano, A., Maric, J.-C., Hoa, D. H. B., Kergoat, M., Portha, B., and Rosselin, G. (1986) *C. R. Acad. Sci. Paris Ser. III* **303,** 357–360
17. Ahrén, B., and Lundquist, I. (1981) *Diabetologia* **20,** 54–59
18. Szecówka, J., Lins, P. E., Tatemoto, K., and Efendic, S. (1983) *Endocrinology* **112,** 1469–1473
19. Yamagami, T., Ohsawa, K., Nishizawa, M., Inoue, C., Gotoh, E., Yanaihara, N., Yamamoto, H., and Okamoto, H. (1988) *Ann. N. Y. Acad. Sci.* **527,** 87–102
20. Lamperti, E. D., Rosen, K. M., and Villa-Komaroff, L. (1991) *Mol. Brain Res.* **9,** 217–231
21. Hanahan, D. (1985) *Nature* **315,** 115–122
22. Lomedico, P., Rosenthal, N., Efstratiadis, A., Gilbert, W., Kolodner, R., and Tizard, R. (1979) *Cell* **18,** 545–558
23. Hogan, B., Constantini, F., and Lacy, E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Unno, M., Yonekura, H., Nakagawara, K., Watanabe, T., Miyashita, H., Morizumi, S., Okamoto, H., Itoh, T., and Teraoka, H. (1993) *J. Biol. Chem.* **268,** 15974–15982
25. Okamoto, H. (1981) *Mol. Cell. Biochem.* **37,** 43–61
26. Sacchi, T. B., Bani, D., and Biliotti, G. (1992) *Pancreas* **7,** 601–610
27. Edwards, R. H., Rutter, W. J., and Hanahan, D. (1989) *Cell* **58,** 161–170
28. Sarvetnick, N., Liggitt, D., Pitts, S. L., Hansen, S. E., and Stewart, T. A. (1988) *Cell* **52,** 773–782
29. Epstein, P. N., Overbeek, P. A., and Means, A. R. (1989) *Cell* **58,** 1067–1073
30. Stewart, T. A., Hultgren, B., Huang, X., Pitts-Meek, S., Hully, J., and MacLachlan, N. J. (1993) *Science* **260,** 1942–1946
31. Epstein, P. N., Boschero, A. C., Atwater, I., Cai, X., and Overbeek, P. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 12038–12042
32. Wahl, M. A., Straub, S. G., and Ammon, H. P. T. (1993) *Diabetologia* **36,** 920–925
33. Hökfelt, T., Johansson, O., Ljungdahl, Å., Lundberg, J. M., and Schultzberg, M. (1980) *Nature* **284,** 515–521
34. Lundberg, J. M., Hedlund, B., and Bartfai, T. (1982) *Nature* **295,** 147–149
35. Holst, J. J., Fahrenkrug, J., Knuhtsen, S., Jensen, S. L., Nielsen, O. V., Lundberg, J. M., and Hökfelt, T. (1987) *Am. J. Physiol.* **252,** G182–G189
36. Rasmussen, H., Zawalich, K. C., Ganesan, S., Calle, R., and Zawalich, W. S. (1990) *Diabetes Care* **13,** 655–666
37. Nishizawa, M., Hayakawa, Y., Yanaihara, N., and Okamoto, H. (1985) *FEBS Lett.* **183,** 55–59
38. Yanaihara, C., Hashimoto, Y., Takeda, Y., Kato, I., Track, N. S., Nokihara, K., Manaka, H., Iwanaga, T., Fujita, T., Okamoto, H., and Yanaihara, N. (1986) *Peptides* **7,** 83–88
39. Ishihara, T., Shigemoto, R., Mori, K., Takahashi, K., and Nagata, S. (1992) *Neuron* **8,** 811–819
40. Gepts, W., and LeCompte, P. M. (1985) in *The Diabetic Pancreas* (Volk, B. W., and Arguilla, E. R., eds) pp. 337–365, Plenum Press, New York
41. Pfeifer, M. A., Halter, J. B., and Porte, D. (1981) *Am. J. Med.* **70,** 579–588
42. Stefan, Y., Orci, L., Malaisse-Lagae, F., Perrelet, A., Patel, Y., and Unger, R. H. (1982) *Diabetes* **31,** 694–700
43. Stribling, R., Brunette, E., Liggitt, D., Gaensler, K., and Debs, R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11277–11281
44. Hyde, S. C., Gill, D. R., Higgins, C. F., Trezise, A. E. O., MacVinish, L. J., Cuthbert, A. W., Ratcliff, R., Evans, M. J., and Colledge, W. H. (1993) *Nature* **362,** 250–255

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 51, Issue of ???? ??, pp. 1–6, 1995
Printed in U.S.A.

Regulatory Role of CD38 (ADP-ribosyl Cyclase/Cyclic ADP-ribose Hydrolase) in Insulin Secretion by Glucose in Pancreatic β Cells

ENHANCED INSULIN SECRETION IN CD38-EXPRESSING TRANSGENIC MICE*

(Received for publication, August 10, 1995, and in revised form, September 20, 1995)

Ichiro Kato‡, Shin Takasawa‡, Atsuya Akabane‡, Osamu Tanaka§, Hiroshi Abe§, Toshinari Takamura‡, Yu Suzuki‡, Koji Nata‡, Hideto Yonekura‡, Takashi Yoshimoto¶, and Hiroshi Okamoto‡‖

From the Departments of ‡Biochemistry and ¶Neurosurgery, Tohoku University School of Medicine, Sendai 980-77, Miyagi, Japan and the §Division of Human Structure and Function, Department of Morphology, Tokai University School of Medicine, Isehara 259-11, Kanagawa, Japan

Cyclic ADP-ribose (cADPR) serves as a second messenger for Ca^{2+} mobilization in insulin secretion, and CD38 has both ADP-ribosyl cyclase and cADPR hydrolase activities (Takasawa, S., Tohgo, A., Noguchi, N., Koguma, T., Nata, K., Sugimoto, T., Yonekura, H., and Okamoto, H. (1993) *J. Biol. Chem.* 268, 26052–26054). Here, we produced transgenic mice overexpressing human CD38 in pancreatic β cells. The enzymatic activity of CD38 in transgenic islets was greatly increased, and ATP efficiently inhibited the cADPR hydrolase activity. The Ca^{2+} mobilizing activity of cell extracts from transgenic islets incubated in high glucose was 3-fold higher than that of the control, suggesting that ATP produced by glucose metabolism increased cADPR accumulation in transgenic islets. Glucose- and ketoisocaproate-induced but not tolbutamide- nor KCl-induced insulin secretions from transgenic islets were 1.7–2.3-fold higher than that of control. In glucose-tolerance tests, the transgenic serum insulin level was higher than that of control. The present study provides the first evidence that CD38 has a regulatory role in insulin secretion by glucose in β cells, suggesting that the Ca^{2+} release from intracellular cADPR-sensitive Ca^{2+} stores as well as the Ca^{2+} influx from extracellular sources play important roles in insulin secretion.

Cyclic ADP-ribose (cADPR)[1] (1) induces the release of Ca^{2+} from microsomes of pancreatic islets (2, 3) and from a variety of other cells (4–10). Glucose raises the cADPR concentration in islets, and cADPR induces insulin secretion from digitonin-permeabilized islets *in vitro* (3). We have therefore suggested that cADPR plays a second messenger role in Ca^{2+} mobilization for insulin secretion (2, 3, 11). Human lymphocyte antigen CD38 (12, 13) has been shown to have both ADP-ribosyl cyclase and cADPR hydrolase activities (11, 14). CD38 was found to be expressed in a variety of tissues and cells including pancreatic islets (11, 15). We have shown that ATP, which is generated during glucose metabolism in islets, inhibits the cADPR hydrolase activity of CD38, thereby increasing the accumulation of cADPR because of decreased destruction (11). CD38 is thus thought to play a central role in glucose-induced insulin secretion in islets. In the present study, we produced transgenic mice overexpressing CD38 in islets and analyzed the subcellular localization of expressed CD38, changes in Ca^{2+} mobilizing activity, and glucose-induced insulin secretion.

EXPERIMENTAL PROCEDURES

Construction of Rat Insulin II Promoter/Human CD38 Hybrid Gene—A rat insulin II promoter previously reported to be active in pancreatic β cells of transgenic mice (16, 17) was employed. The 0.7-kilobase pair *Bam*HI–*Xma*I fragment (17) of the rat insulin II promoter (nucleotides −695 to +22 in Ref. 18), the 0.9-kilobase pair *Xma*I–*Sal*I fragment of the human CD38 cDNA (11, 13) (nucleotides +58 to +980 in Ref. 13; *Xma*I and *Sal*I sites introduced by polymerase chain reaction), and the 1.6-kilobase pair *Sal*I–*Eco*RI fragment of the SV40 intron and polyadenylation signal (19) (*Sal*I site derived from plasmid sequence) were ligated at the *Xma*I and *Sal*I sites in the correct orientation. The resultant hybrid gene (Ins-CD38; 3.2 kilobase pairs) was separated from the plasmid vector pBlueScript SK− (Stratagene) by *Kpn*I and *Not*I and was microinjected into fertilized eggs as described (17).

Northern Blot Analyses—Northern blot analyses were carried out on total RNA extracted from various tissues as described (17) using a ^{32}P-labeled human CD38 cDNA probe (*Eco*RI–*Eco*RI fragment of 528 base pairs; nucleotides +29 to +556 in Ref. 13). Hybridization signals were scanned with a bioimage analyzer, BAS 2000 (Fuji Photo Co., Ltd., Tokyo, Japan).

Immunohistochemical Analysis—Immunohistochemical analysis was carried out as described (17) using the diluted (1:20) monoclonal antibody to human CD38 (T16; Cosmo Bio, Japan) and an avidin-biotin peroxidase kit (Vector, Burlingame, CA).

Measurement of cADPR and ADPR Formations from NAD^+—Transgenic (lines 18 and 56) and nontransgenic islets were isolated in parallel from 6–10-week-old litters by the collagenase digestion method (20). Five hundred islets were sonicated at 4 °C for 15 s in 0.1 ml of the homogenizing buffer (20 mM Hepes, pH 7.2, containing 1 mM $MgCl_2$, 0.1 mM EGTA, and 10 μg/ml aprotinin). Formations of cADPR and ADPR from NAD^+ were measured as described (11, 21); briefly, the islet cell homogenate (10 μg of protein) was incubated for 10 min at 37 °C in 0.1 ml of phosphate-buffered saline (pH 7.4) with 0.2 mM NAD^+ containing 5 μCi of [^{32}P]NAD^+ (DuPont NEN). Reaction products were analyzed by high pressure liquid chromatography (HPLC) using a flow scintillation analyzer (Flow-One Beta-525TR, Packard, Meriden, CT).

Subcellular Fractionation and Measurement of NAD^+ Glycohydrolase Activity—The subcellular fractionation was performed according to the islet fractionation method of McDaniel *et al.* (22). One thousand islets were homogenized in 400 μl of homogenizing buffer (50 mM MES, 1 mM EDTA, and 0.25 M sucrose, pH 7.2). The homogenate was centrifuged at 600 × *g* for 5 min to yield a pellet containing nuclei (nuclear fraction). Centrifugation of the supernatant at 20,000 × *g* for 20 min yielded a pellet containing the plasma membrane, secretory granules,

* This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan and the Uehara Memorial Foundation, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Dept. of Biochemistry, Tohoku University School of Medicine, 2-Seiryo-machi, Aoba-ku, Sendai 980-77, Miyagi, Japan. Tel.: 81-22-274-1111, ext. 2211; Fax: 81-22-272-8101.

[1] The abbreviations used are: cADPR, cyclic ADP-ribose; ADPR, ADP-ribose; HPLC, high pressure liquid chromatography; KIC, ketoisocaproate; MES, 4-morpholineethanesulfonic acid.

and mitochondria (membrane fraction). Centrifugation of the resultant supernatant at 150,000 × *g* for 90 min yielded a pellet containing the microsome (microsome fraction). The supernatant after 150,000 × *g* centrifugation yielded a soluble protein (cytosol fraction). NAD^+ glycohydrolase activity in the fractionated protein was measured as described (23). Briefly, 1 μg of each fractionated protein was incubated with 0.2 mM NAD^+ containing 50 nCi of [^{14}C]NAD^+ (Amersham Corp.) at 37 °C for 10 min. The mixture was applied on a column of Dowex-1 (Bio-Rad Laboratories). Nicotinamide was eluted with 20 mM Tris-HCl (pH 7.5), followed by scintillation counting.

Measurement of Insulin Secretion from Isolated Islets—Islets of transgenic mice (line 18) and control mice were isolated in parallel from 6–10-week-old litters by collagenase digestion. Twenty islets were incubated for 1 h at 37 °C in 1 ml of RPMI 1640 medium containing 10% fetal calf serum and various concentrations of glucose. The medium samples were subsequently assayed for radioimmunoassay of insulin using the insulin radioimmunoassay kit (Amersham Corp.) and rat insulin standards. For the time course experiment, 20 islets were incubated at 37 °C in 1 ml of the medium containing 11.1 mM glucose, and medium samples (2 μl) collected at 10, 20, 30, and 40 min after the incubation were subjected to radioimmunoassay for insulin. For measurements of insulin secretion by other insulin secretagogues, 10 islets were incubated for 1 h at 37 °C in 0.5 ml of the medium containing the lowest concentration of glucose (2.5 mM) and then incubated for another 1 h in the same medium containing 10 mM ketoisocaproic acid (KIC) (Sigma), 0.2 mM tolbutamide (Sigma), or 25 mM KCl (Merck). The medium samples were subsequently radioimmunoassayed for insulin.

Assay of Ca^{2+} Mobilizing Activity—Five hundred transgenic islets (line 18) or control islets were incubated at 37 °C for 15 min in 5 ml of RPMI 1640 medium containing 10% fetal calf serum and 2.5 or 11.1 mM glucose. After the incubation, islet cell extracts (50 μl) were prepared as described (3). Release of Ca^{2+} was monitored by adding the islet extracts (15–20 μl) to 3 ml of intracellular medium (3) containing 3 μM fluo 3, a fluorescent Ca^{2+} indicator, and the rat cerebellum microsome fraction (88 μg of protein) prepared as described (3). Assay of Ca^{2+} mobilizing activity using the mouse islet microsome fraction (5 μg of protein) was carried out with 0.6 ml of the intracellular medium (see Fig. 6). Fluorescence was measured at 490-nm excitation and 535-nm emission at 37 °C.

Measurement of cADPR Hydrolase Activity in the Presence of Various Concentrations of ATP—The islet cell homogenate (10 μg of protein) of transgenic mice (line 18) was incubated for 20 min at 37 °C in 0.1 ml of phosphate-buffered saline (pH 7.4) in the presence of 0–6 mM ATP and 0.2 mM cADPR containing 5 μCi of [^{32}P]cADPR, prepared enzymatically from NAD^+ and [^{32}P]NAD^+ using *Aplysia kurodai* ADP-ribosyl cyclase. Reaction products were analyzed by HPLC (11, 21).

Measurement of Serum Insulin and Blood Glucose Levels in Glucose Tolerance Tests—Transgenic mice (lines 18 and 56) and their respective nontransgenic siblings were fasted 10 h and then subjected to glucose tolerance tests by an intraperitoneal injection of 1 g of glucose/kg of body weight. Blood samples (100 μl) were taken from the tail vein at each point after glucose administration, and the serum samples (25 μl) were prepared by centrifugation after incubating blood samples overnight at 4 °C to complete coagulation. The serum insulin levels were determined by radioimmunoassay. Blood glucose determinations were made on fresh whole blood (15 μl) using the Accucheck II (Boehringer Mannheim). All statistical analyses were performed using Student's *t* test.

RESULTS AND DISCUSSION

The rat insulin II promoter/human CD38 hybrid gene (Ins-CD38; see "Experimental Procedures") was designed to direct the overexpression of CD38 in pancreatic β cells of transgenic mice. The linearized gene fragment was microinjected into the fertilized eggs of (C57Bl/6J × CBA/J) F_1 mice. 20 out of 94 newborn mice were found to carry the Ins-CD38 transgene, as detected by polymerase chain reaction analyses using primers for the insulin promoter and human CD38 cDNA. In the present study, the six transgenic lines, 18, 30, 49, 56, 60, and 72, were maintained on ICR background and analyzed.

Northern blot analysis using the human CD38 cDNA probe (11) showed that all lines of transgenic mice but not that of the nontransgenic mice expressed human CD38 mRNA in the pancreatic islets (Fig. 1*A*). Densitometric scanning indicated that the transgenic lines 18, 56, and 60 expressed relatively higher

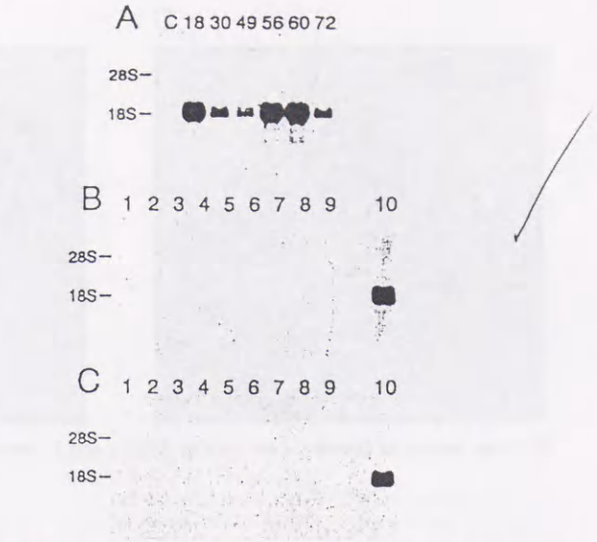


FIG. 1. **Northern blot analyses of RNAs from pancreatic islets (*A*) and from various tissues of transgenic mouse line 18 (*B*) and line 56 (*C*) using human CD38 cDNA.** *A*, total RNAs (1.5 μg/lane) isolated from pancreatic islets of control nontransgenic mice (*C*), transgenic line 18 (*18*), line 30 (*30*), line 49 (*49*), line 56 (*56*), line 60 (*60*), and line 72 (*72*) were used for RNA blot hybridization. *B* and *C*, *lanes 1*, brain; *lanes 2*, lung; *lanes 3*, heart; *lanes 4*, stomach; *lanes 5*, small intestine; *lanes 6*, liver; *lanes 7*, kidney; *lanes 8*, spleen; *lanes 9*, testis (*lanes 1–9*, 10 μg of RNA/lane). *Lane 10*, pancreatic islets (1.5 μg of RNA/lane). Positions of mouse 28 S and 18 S rRNAs are presented on the *left*. The endogenous mouse CD38 mRNA did not hybridize under the conditions used in this study.

levels of human CD38 mRNA in islets, whereas lines 30, 49, and 72 expressed lower levels of human CD38 mRNA. The human CD38 mRNA expression was not detected in other tissues such as brain, lung, heart, stomach, small intestine, liver, kidney, spleen, and testis in the transgenic mice (Fig. 1, *B* and *C*), indicating that the expression of human CD38 is limited to islets. In immunohistochemistry, islets of the transgenic mice were densely stained for human CD38 (Fig. 2, *B* and *C*). On the other hand, islets of the control mice showed no immunoreactivity for human CD38 (Fig. 2*A*). In contrast to islets, the pancreatic exocrine cells showed no detectable staining for human CD38 in any of the transgenic and nontransgenic mice.

Next, the pancreatic islet homogenates prepared from islets of transgenic and nontransgenic siblings of lines 18 and 56 were incubated with [^{32}P]NAD^+, and the reaction products were analyzed by HPLC. The formation of cADPR in the transgenic mice (for line 18, 1.5 nmol/min·mg protein; for line 56, 1.5 nmol/min·mg protein) was indeed much higher than in the controls (<0.05 nmol/min·mg protein). The formation of ADPR in the transgenic mice (for line 18, 72.8 nmol/min·mg protein; for line 56, 174.4 nmol/min·mg protein) was also much higher than in the controls (4.3 nmol/min·mg protein). CD38 exhibits both ADP-ribosyl cyclase and cADPR hydrolase activities, and the overall reaction is classified as an NAD^+ glycohydrolase reaction (11, 14). To determine the subcellular distribution of expressed CD38, islet proteins were fractionated by centrifugation (22) and fractionated proteins were assayed for NAD^+ glycohydrolase activity (Table I). The NAD^+ glycohydrolase activity in the membrane fraction was greatly increased in the transgenic mice, indicating that expressed CD38 was predominantly localized in this fraction. Significant activities were also detected in the nuclear, microsome and cytosol fractions of the transgenic islets. The distribution of the percentage of total activity in fractions of transgenic mice showed a similar tend-

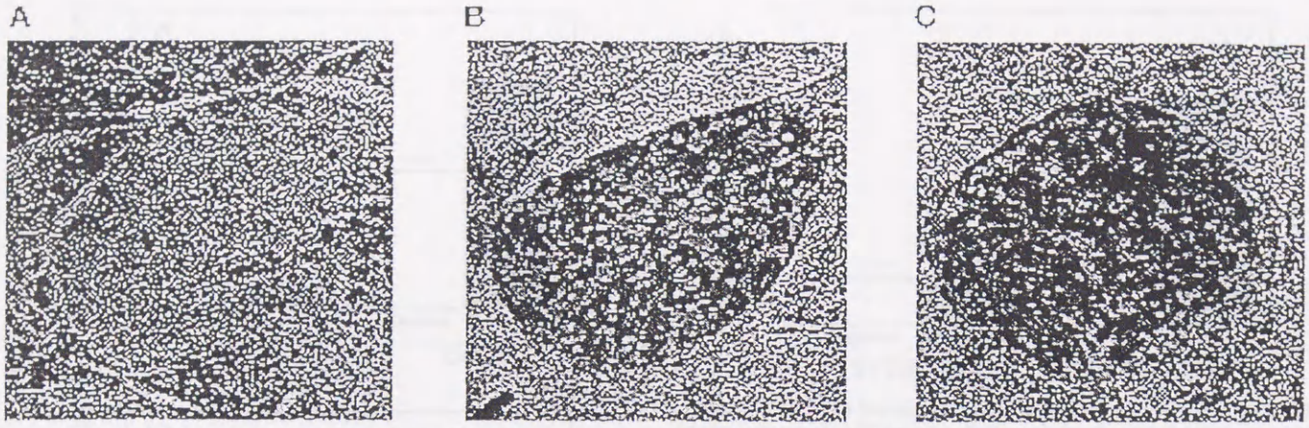


FIG. 2. **Immunohistochemical detection of human CD38 in mouse pancreas.** Human CD38 protein was detected in mouse islets of transgenic line 18 (*B*) and line 56 (*C*) but not in nontransgenic mouse (*A*).

TABLE I
Subcellular distribution of NAD⁺ glycohydrolase activity

Transgenic and control islet homogenates were fractionated and assayed for NAD^+ glycohydrolase activity as described under "Experimental Procedures." Specific activities are expressed as nmol/min/mg of protein. Total NAD^+-glycohydrolase activity was determined for each fraction and expressed as the percentage of activity to homogenate total activity.

Fraction	Control		Line 18		Line 56	
	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
		%		%		%
Homogenate	21.9	100	181.1	100	155.6	100
Nuclear	22.6	48.1	123.1	23.1	104.3	30.3
Membrane	12.3	24	270.3	67.7	244.5	51.1
Microsome	16.6	3.4	101.5	3.9	191.7	8.4
Cytosol	2.9	1.7	51.8	3.9	35.9	2.8

ency to that of the control.

We isolated islets from transgenic mouse line 18 and their nontransgenic litter mates and measured secreted insulin after incubation in medium containing various concentrations of glucose (Fig. 3*A*). At 6.8–15.6 mM glucose, the transgenic insulin secretion was 1.7–2.3-fold higher than that of the control. Essentially similar results were obtained using transgenic line 56 (data not shown). Time course experiments at 11.1 mM glucose indicated that at 10 min after the exposure to glucose, the glucose-stimulated insulin secretion was significantly higher in the transgenic islets and progressively increased in a time-dependent manner (Fig. 3*B*).

We next investigated the effects of other insulin secretagogues on insulin secretion from transgenic and control islets. When the islets were exposed to 10 mM KIC, which, like glucose, generates ATP during the metabolism (24), the transgenic insulin secretion was 1.7-fold higher than that of the control (Fig. 4*A*). Tolbutamide blocks ATP-sensitive K^+ channel and facilitates Ca^{2+} influx through voltage-dependent Ca^{2+} channels (24) without increasing the islet ATP concentration (25). When the islets were exposed to 0.2 mM tolbutamide, the transgenic insulin secretion was not altered as compared with the control (Fig. 4*B*). When the islets were exposed to 25 mM KCl, which directly induces cell membrane depolarization resulting in Ca^{2+} influx (24), the transgenic insulin secretion was also not significantly higher than the control (Fig. 4*C*).

We prepared cell extracts from the islets incubated in 2.5 or 11.1 mM glucose. The extracts were assayed for the Ca^{2+} mobilizing activity from microsomes (3). The Ca^{2+} mobilizing activity of extracts of transgenic islets incubated in 11.1 mM glucose was 3-fold higher than that of the control extracts (Fig. 5). The Ca^{2+} mobilization by the islet extracts was abolished when the microsomes had been desensitized by previously releasing

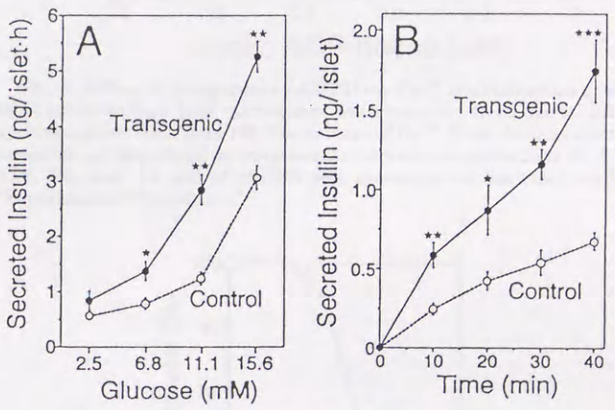


FIG. 3. **Glucose-induced insulin secretion from isolated islets.** *A*, insulin secretion from isolated islets under various glucose concentrations. The results shown are the averages from 10–25 assays performed on five different preparations of transgenic (line 18) and control islets. *B*, time course of insulin secretion from isolated islets under 11.1 mM glucose. $n = 5$ for each point. *Vertical bars* indicate S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Ca^{2+} in response to authentic cADPR (3), indicating that Ca^{2+} mobilization by the extracts of transgenic islets after high glucose treatment is cADPR-derived. In contrast, at 2.5 mM glucose, the Ca^{2+} mobilizing activities of the transgenic and control islet extracts were lower, and there was no significant difference between the two extracts (Fig. 5). The effect of exogenous cADPR on Ca^{2+} mobilization from control and transgenic islet microsomes was essentially similar (Fig. 6), suggesting that microsome sensitivity to cADPR is not altered by human CD38 overexpression.

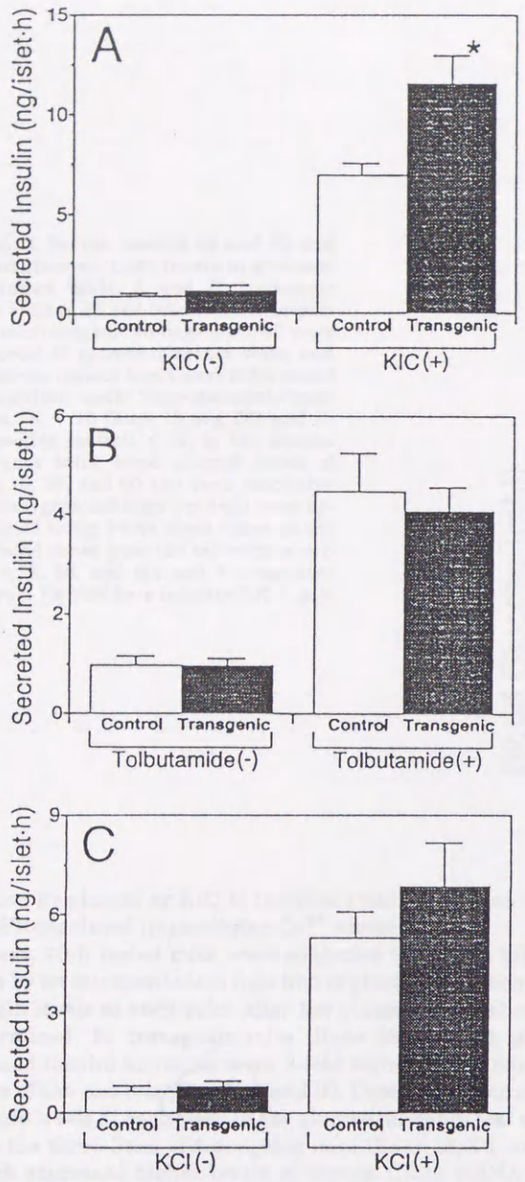


FIG. 4. **Insulin secretion from isolated islets by KIC, tolbutamide, and KCl.** Insulin secretion from islets of control mice and transgenic mice (line 18) stimulated by 10 mM KIC (*A*), 0.2 mM tolbutamide (*B*), or 25 mM KCl (*C*) was measured as described under "Experimental Procedures." *Open bars* and *shaded bars* show levels from control mice and transgenic mice (line 18), respectively. $n = 5$ for each point. *Vertical bars* indicate S.E. *, $p < 0.01$.

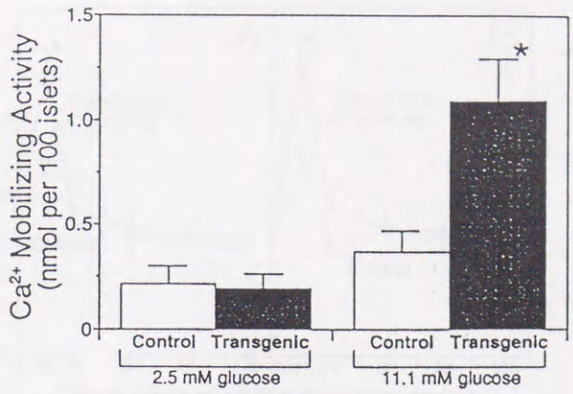


FIG. 5. **Ca^{2+} mobilizing activity in the transgenic and control islets.** Release of Ca^{2+} from cerebellar microsomes by the islet extracts prepared after incubation with 2.5 or 11.1 mM glucose was measured as described under "Experimental Procedures." *Open bars* and *shaded bars* show levels from control mice and transgenic mice (line 18), respectively. $n = 3$ for each mouse. *Vertical bars* indicate S.E. *, $p < 0.05$.

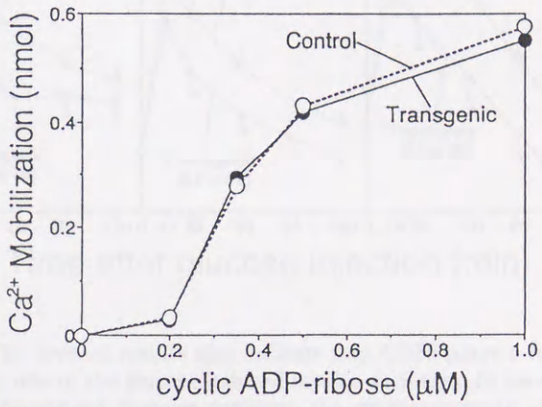


FIG. 6. **Effect of exogenous cADPR on Ca^{2+} mobilization from islet microsomes.** Islet microsomes were prepared from control mice and transgenic mice (line 18). The release of Ca^{2+} from the islet microsomes (5 μg of protein) in response to various concentrations (0, 0.2, 0.35, 0.5, and 1.0 μM) of cADPR was measured as described under "Experimental Procedures."

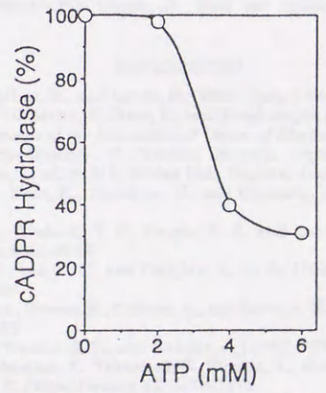


FIG. 7. **Effects of ATP on cADPR hydrolase activity of expressed CD38.** ADPR synthesis from cADPR by islet cell homogenate of transgenic mice (line 18) in the presence of 0, 2, 4, or 6 mM ATP was measured as described under "Experimental Procedures."

We have previously shown that ATP, generated during glucose metabolism in islets, dose-dependently inhibits the cADPR hydrolyzing activity of CD38 expressed in COS-7 cells and increases the accumulation of cADPR (11). In fact, higher concentrations of ATP efficiently inhibited the cADPR hydrolase activity of the CD38 expressed in transgenic islets (Fig. 7). The cellular ATP concentration[2] in transgenic islets increased from 2.3 (at 2.5 mM glucose) to 3.3 mM (at 11.1 mM glucose); this rise in cellular ATP concentrations would inhibit the cADPR hydrolase activity of CD38 and thereby increase the cADPR concentration. It is therefore reasonable to assume that the expressed ADP-ribosyl cyclase/cADPR hydrolase (CD38) in transgenic islets generates the enhanced cADPR accumulation upon stim-

[2] Thirty islets were incubated for 15 min in the presence of 2.5 or 11.1 mM glucose. After the incubation, the ATP level was determined by a bioluminescence assay procedure using an ATP monitoring kit (Bio Orbit, Turku, Finland) as described (30). The ATP concentrations (in mM) were calculated on the basis of the mean diameter (220 μm) of the islets, which was measured microscopically. The ATP concentrations obtained in the present study were comparable to those reported previously (1.8–3.9 mM in Ref. 25).

Orig. Op.	OPERATOR:	Session	PROOF:	PE's:	AA's:	COMMENTS:	ARTNO:
1st K82, 2nd	judy	5					356768

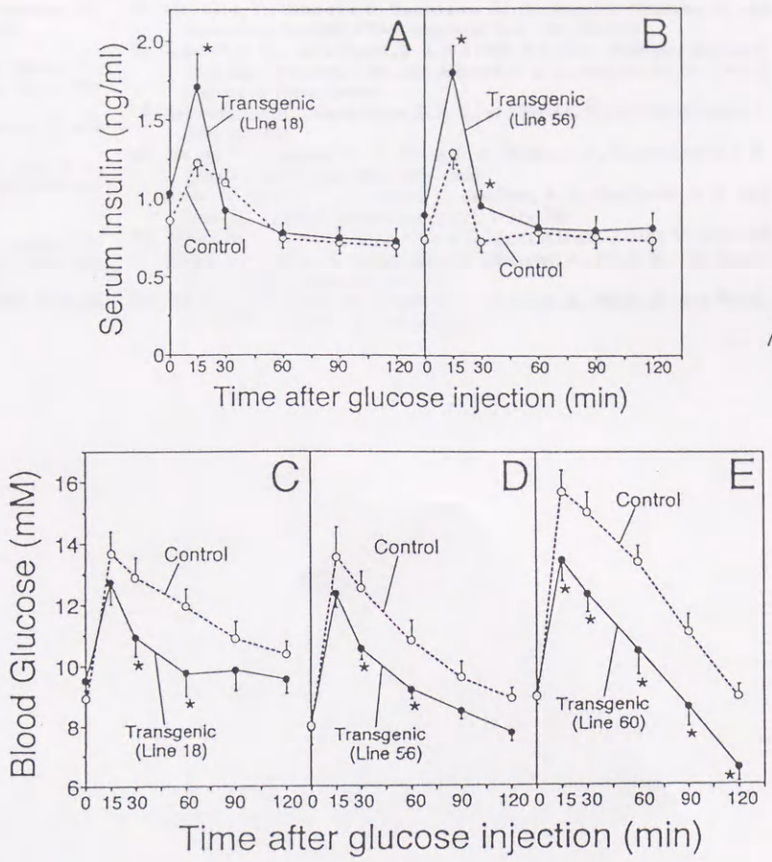


FIG. 8. **Serum insulin (*A* and *B*) and blood glucose (*C–E*) levels in glucose-tolerance tests.** *A* and *B*, transgenic mice of lines 18 and 56 and their respective nontransgenic siblings (*control*) were subjected to glucose-tolerance tests, and the serum insulin levels were determined as described under "Experimental Procedures." $n = 10$ (lines 18 and 56) and 10 (respective control). *C–E*, in the glucose tolerance tests, blood glucose levels of lines 18, 56, and 60 and their respective nontransgenic siblings (*control*) were determined using whole blood taken at the indicated times from the tail vein. $n = 8$ (lines 18, 56, and 60) and 8 (respective control). *Vertical bars* indicate S.E. *, $p < 0.05$.

ulation by glucose or KIC to increase insulin secretion via the cADPR-mediated intracellular Ca^{2+} elevation.

Next, 10-h fasted mice were subjected to glucose tolerance tests by an intraperitoneal injection of glucose, and then serum insulin levels at each point after the glucose stimulation were determined. In transgenic mice (lines 18 and 56), glucose-induced insulin increases were 2-fold higher (at 15 min) than those of the controls (Fig. 8, *A* and *B*). Determinations of blood glucose levels at each point in the glucose tolerance test showed that the three lines of transgenic mice (lines 18, 56, and 60), which expressed higher levels of human CD38 mRNA in the pancreatic islets (Fig. 1*A*), had lower glucose levels than the controls (Fig. 8, *C–E*). However, transgenic lines 30, 49, and 72, which expressed lower levels of human CD38 mRNA in islets, did not have significantly lower glucose levels after glucose administration than the controls. These results indicate that the expressed ADP-ribosyl cyclase/cADPR hydrolase (CD38) reproducibly and dose-dependently facilitates glucose-induced insulin secretion *in vivo* and, by doing so, reduces the blood glucose levels of the transgenic mice.

It should be noted that the production of human CD38 in islets did not appear to be deleterious to the health of the transgenic mice; fertility and body weight were indistinguishable from controls. The islets of transgenic mice at 1 year of age appeared morphologically normal and were well stained for insulin (not shown).

The results of the present study suggest that not only Ca^{2+} from extracellular sources (Ca^{2+} influx through voltage-dependent Ca^{2+} channels evoked by glucose-induced cell membrane depolarization; Ref. 24) but also Ca^{2+} released from intracellular stores (cADPR-induced Ca^{2+} release from microsomes; Ref. 3) play important roles in regulating the glucose-induced insulin secretion. In fact, intracellular Ca^{2+} elevation in the absence of external Ca^{2+} has been reported recently (26, 27).

The present results also indicate that CD38 plays a regulatory role in the glucose-induced insulin secretion. In non-insulin-dependent diabetes mellitus, the glucose-induced insulin secretion is impaired (28) even when pancreatic islets retain significant amounts of insulin (29). Thus, it would be important to determine whether there are qualitative or quantitative differences in the CD38 (ADP-ribosyl cyclase/cADPR hydrolase) in non-insulin-dependent diabetes mellitus β cells.

Acknowledgment—We thank B. Bell for critical reading of the manuscript.

REFERENCES

1. Lee, H. C., Aarhus, R., and Levitt, D. (1994) *Nature Struct. Biol.* **1**, 143–144
2. Okamoto, H., Takasawa, S., Nata, K., and Yonekura, H. (1992) *Abstracts of the First Conference of the International Union of Biochemistry and Molecular Biology: Biochemistry of Diseases, Nagoya, Japan, June 1–6, 1992* (Yamashina, I., ed) p. 218, Bridge Ltd., Nagoya, Japan
3. Takasawa, S., Nata, K., Yonekura, H., and Okamoto, H. (1993) *Science* **259**, 370–373
4. Clapper, D. L., Walseth, T. F., Dargie, P. J., and Lee, H. C. (1987) *J. Biol. Chem.* **262**, 9561–9568
5. Koshiyama, H., Lee, H. C., and Tashjian, A. H., Jr. (1991) *J. Biol. Chem.* **266**, 16985–16988
6. Currie, K. P. M., Swann, K., Galione, A., and Scott, R. H. (1992) *Mol. Biol. Cell* **3**, 1415–1422
7. White, A. M., Watson, S. P., and Galione, A. (1993) *FEBS Lett.* **318**, 259–263
8. Hua, S.-Y., Tokimasa, T., Takasawa, S., Furuya, Y., Nohmi, M., Okamoto, H., and Kuba, K. (1994) *Neuron* **12**, 1073–1079
9. Sasaki, T., Shimura, S., Takasawa, S., Nagaki, M., Nara, M., Okamoto, H., and Shirato, K. (1993) *Am. Rev. Respir. Dis.* **147**, 936 (abstr.)
10. Higashida, H., Robbins, J., Egorova, A., Noda, M., Taketo, M., Ishizaka, N., Takasawa, S., Okamoto, H., and Brown, D. A. (1995) *J. Physiol. (Lond.)* **482**, 317–323
11. Takasawa, S., Tohgo, A., Noguchi, N., Koguma, T., Nata, K., Sugimoto, T., Yonekura, H., and Okamoto, H. (1993) *J. Biol. Chem.* **268**, 26052–26054
12. Reinherz, E. L., Kung, P. C., Goldstein, G., Levey, R. H., and Schlossman, S. F. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 1588–1592
13. Jackson, D. G., and Bell, J. I. (1990) *J. Immunol.* **144**, 2811–2815
14. Howard, M., Grimaldi, J. C., Bazan, J. F., Lund, F. E., Santos-Argumedo, L., Parkhouse, R. M. E., Walseth, T. F., and Lee, H. C. (1993) *Science* **262**, 1056–1059

15. Koguma, T., Takasawa, S., Tohgo, A., Karasawa, T., Furuya, Y., Yonekura, H., and Okamoto, H. (1994) *Biochim. Biophys. Acta* **1223**, 160–162
16. Hanahan, D. (1985) *Nature* **315**, 115–122
17. Kato, I., Suzuki, Y., Akabane, A., Yonekura, H., Tanaka, O., Kondo, H., Takasawa, S., Yoshimoto, T., and Okamoto, H. (1994) *J. Biol. Chem.* **269**, 21223–21228
18. Lomedico, P., Rosenthal, N., Efstratiadis, A., Gilbert, W., Kolodner, R., and Tizard, R. (1979) *Cell* **18**, 545–558
19. Reddy, V. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Ghosh, P. K., Celma, M. L., and Weissman, S. M. (1978) *Science* **200**, 494–502
20. Okamoto, H. (1981) *Mol. Cell. Biochem.* **37**, 43–61
21. Tohgo, A., Takasawa, S., Noguchi, N., Koguma, T., Nata, K., Sugimoto, T., Furuya, Y., Yonekura, H., and Okamoto, H. (1994) *J. Biol. Chem.* **269**, 28555–28557
22. McDaniel, M. L., Colca, J. R., Kotagal, N., and Lacy, P. E. (1983) *Methods Enzymol.* **98**, 182–200
23. Karasawa, T., Takasawa, S., Yamakawa, K., Yonekura, H., Okamoto, H., and Nakamura, S. (1995) *FEMS Microbiol. Lett.* **130**, 201–204
24. Ashcroft, F. M., and Ashcroft, S. J. H. (1992) in *Insulin: Molecular Biology to Pathology* (Ashcroft, F. M., and Ashcroft, S. J. H., eds) pp. 97–150, Oxford University Press, Oxford
25. Ashcroft, S. J. H., Weerasinghe, L. C. C., and Randle, P. J. (1973) *Biochem. J.* **132**, 223–231
26. Roe, M. W., Lancaster, M. E., Mertz, R. J., Worley, J. F., III, and Dukes, I. D. (1993) *J. Biol. Chem.* **268**, 9953–9956
27. Rojas, E., Carroll, P. B., Ricordi, C., Boschero, A. C., Stojilkovic, S. S., and Atwater, I. (1994) *Endocrinology* **134**, 1771–1781
28. Pfeifer, M. A., Halter, J. B., and Porte, D., Jr. (1981) *Am. J. Med.* **70**, 579–588
29. Stefan, Y., Orci, L., Malaisse-Lagae, F., Perrelet, A., Patel, Y., and Unger, R. H. (1982) *Diabetes* **31**, 694–700
30. Sandler, S., Bendtzen, K., Eizirik, D. L., Strandell, E., Welsh, M., and Welsh, N. (1990) *Immunol. Lett.* **26**, 245–252

Orig. Op.	OPERATOR:	Session	PROOF:	PE's:	AA's:	COMMENTS:	ARTNO:
1st 8000, 2nd	judy	5	SPKW				356768

11. 膵ランゲルハンス島β細胞でヒトVIP遺伝子を過剰発現するトランスジェニックマウスの作製と導入遺伝子による血糖降下作用

加藤 一郎[*1] 鈴木 雄[*1] 赤羽 敦也[*1]
田中 理[*2] 近藤 尚武[*2] 米倉 秀人[*1]
岡本 宏[*1]

はじめに

VIP (Vasoactive Intestinal Peptide) は，C末端がアミド化された28アミノ酸よりなるペプチドであり当初腸管より単離された[1]。VIPは構造・機能がよく似たもう1つのペプチドであるPHM-27 (peptide histidine methionine-27) とともに一つの大きな前駆体蛋白として合成された後[2]，プロセシングによりそれぞれが同じモル数生成される。VIPは腸管だけでなく中枢・末梢神経系や内分泌組織など全身に広く分布し，その生理作用は血管拡張・血圧降下作用の他に，腸管からの水分・電解質の分泌刺激，気管支・腸管での平滑筋弛緩作用，脳血管の血流調節作用などの多岐にわたる[3]。

VIPは膵灌流実験でインスリン分泌を促進すること[4]，また免疫組織染色で膵臓の神経内に見出されること[5]から，神経終末より放出されて膵ランゲルハンス島細胞の機能を制御する神経伝達物質としての可能性が示唆されてきた。しかしながらヒト[5]やマウス[6]において，VIPを経静脈的に投与すると確かにインスリン分泌は促進されるものの血糖は不変であり，またVIPomaの患者ではVIPのグリコーゲン分解作用によると考えられる高血糖が高頻度に観察される[7]。従って，VIPが実際に *in vivo* においてどのように膵ランゲルハンス島のインスリン分泌を介して全身の血糖調節に効果を及ぼしているかは不明であった。

今回，われわれはヒトVIP遺伝子導入トランスジェニックマウスのphenotypeを解析することにより，VIPにより高濃度グルコース刺激時のインスリン分泌反応の増強・それによる耐糖能改善がもたらされること，従って将来的にVIPを糖尿病治療の薬剤として使用し得る可能性があることを明らかにした。

方法

1. トランスジェニックマウスの作製

ヒトVIP遺伝子を膵β細胞特異的に発現させるためラットインスリンプロモーター (0.7kb) の下流にヒトVIP遺伝子[8]の全長 (9.8kb) を連結し，インスリン/VIPキメラ遺伝子 (Fig. 1) を作製した。このDNA断片 (10.5kb) をBamHIによりプラスミドより切りだし，マウス受精卵雄性

Transgenic mice overexpressing human vasoactive intestinal peptide gene in pancreatic β cells and hypoglycemic effect of the transgene

Ichiro Kato[*1], Yu Suzuki[*1], Atsuya Akabane[*1], Osamu Tanaka[*2], Hisatake Kondo[*2], Hideto Yonekura[*1] and Hiroshi Okamoto[*1]

Departments of Biochemistry[*1] and Anatomy[*2], Tohoku University School of Medicine

[*1] 東北大学医学部第一医化学 〒980 仙台市青葉区星陵町2-1 (022-274-1111)

[*2] 東北大学医学部第二解剖学 同上

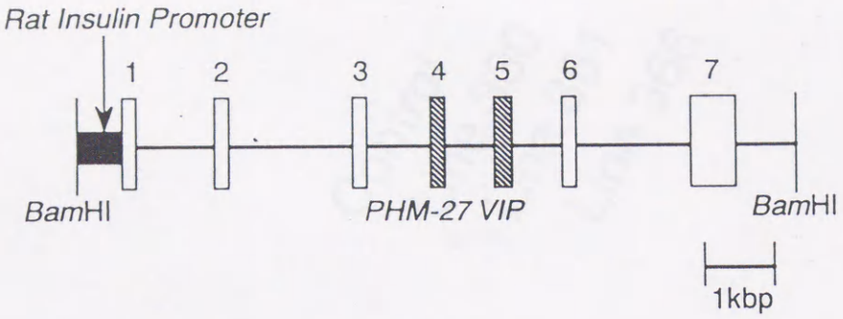


Fig. 1 Schematic representation of the rat insulin II/human VIP fusion gene used for microinjection. The transgene (10.5 kbp) consists of the rat insulin II promoter (0.7kbp) and the entire human VIP gene (9.8kbp) extending from 25 basepairs of the 5′-noncoding region of exon 1 up to 0.9 kbp of the 3′-flanking region.

前核に顕微鏡下注入した。この受精卵を偽妊娠マウスの卵管に移植した。出生したマウスの尻尾よりゲノム DNA を抽出し，Polymerase chain reaction (PCR) 法によりトランスジェニックマウスを同定した。以降は ICR マウスと交配させ，F_1，F_2 を用いて解析を行った。

2.ノーザンブロット解析

コントロールマウス，トランスジェニックマウスの膵臓より total RNA を抽出，各レーン 5μg を電気泳動し，ナイロンフィルターにトランスファーした。ヒト VIP cDNA フラグメントをランダムプライム法を用いてラベリングし，それをプローブとしてハイブリダイゼーションし，洗浄した後，オートラジオグラフィーを行った。

3.膵臓の免疫組織染色

マウスの膵臓をホルマリン固定した後，VIP 抗体 (Amersham)，PHM-27 抗体 (Affinity Research Products)，インスリン抗体 (Dako) を一次抗体として，avidin-biotin peroxidase 法 (Vector Laboratories) により免疫組織染色を行った。

4.単離膵島からの VIP 分泌量の測定

コントロールマウスおよびトランスジェニックマウスの膵臓よりコラゲナーゼ法により単離した 100 個のランゲルハンス島を，2.7mM または 16.7mM glucose 含有の RPMI 1640/10% fetal calf serum 中で培養し，経時的にサンプリングして培地中に分泌された VIP をラジオイムノアッセイ法により定量した。

5.VIP・インスリンの定量

VIP については Amersham のキットを用い，ヒト VIP をスタンダードとしてラジオイムノアッセイを行った。インスリンについては Novo Nordisk Biolabs のキットを用い，ラットインスリンをスタンダードとしてラジオイムノアッセイを行った。

6.糖負荷試験

12 時間マウスを絶食させた後，体重 1g 当たり 2mg のグルコースをマウス腹腔内に注射して糖負荷を与えた。経時的にマウス尻尾より採血し AccucheckII (Boehringer Manheim) を用いて血糖値を測定した。70% 膵切除マウスは，ネンブタール麻酔下に膵の splenic portion および gastric portion を外科的に切除して作製し，膵切除後 1 週間後に同様に糖負荷試験を行った。

結　果

1.ヒト VIP 遺伝子導入トランスジェニックマウスの作製と膵臓における導入遺伝子の発現

マウスのゲノム DNA を PCR 法によりスクリーニングした結果，仔マウス 69 匹中 4 匹がトランスジェニックマウスでありそのうち遺伝子を子孫に伝達したものは 3 匹であった。膵 RNA を用いたノーザンブロット解析 (Fig. 2) の結果，3 ライ

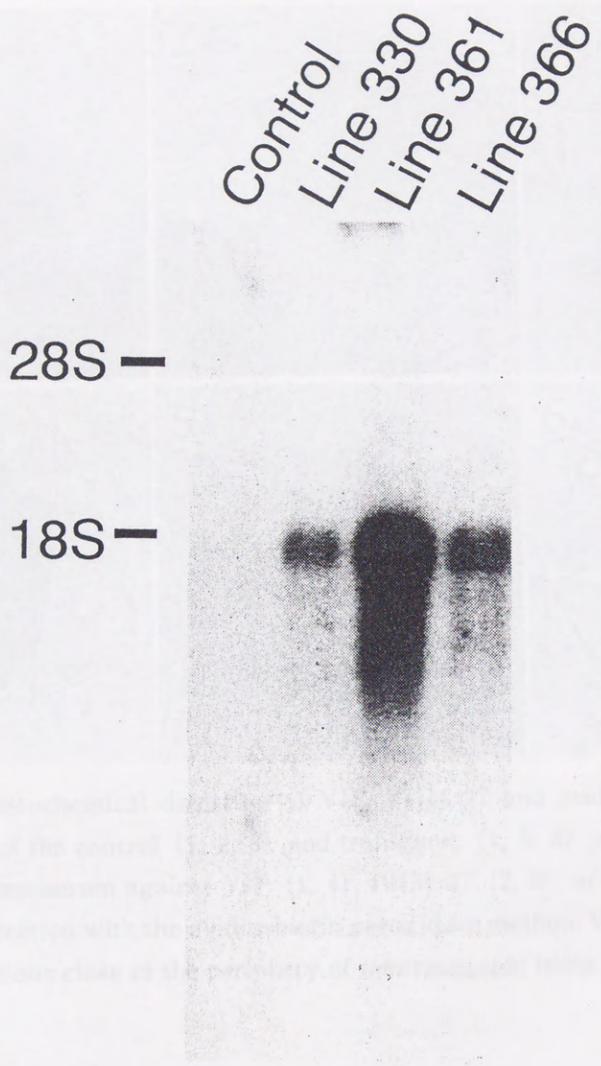


Fig. 2 Northern blot analysis of pancreas total RNA from transgenic mice and control mice. RNA (5μg) was electrophoresed and transferred to a nylon membrane. The membrane was hybridized with the human VIP cDNA probe, washed and exposed to x-ray film at −80°C.

ンの全てでヒトVIP遺伝子の転写が認められた。ICRマウス由来のライン330について以下全ての解析を行った。ゲノミックサザンブロット解析ではライン330においては1ハプロイド当たり8コピーのヒトVIP遺伝子の導入が認められた。

2. トランスジェニックマウスの膵ランゲルハンス島β細胞でVIPおよびPHM-27が産生・分泌される

VIP抗体によって膵ランゲルハンス島自身は染色されないが，近傍の神経線維や神経節にVIPが存在している（Fig. 3-1）。これに対し，トランスジェニックマウスの膵ランゲルハンス島においては導入遺伝子由来のVIP蛋白の産生が見出された（Fig. 3-4）。PHM-27に対する抗体で免疫組織染色を行うと，トランスジェニックマウスの膵ランゲルハンス島においてPHM-27蛋白の産生が認められた（Fig. 3-5）。これはPHM-27がヒトVIP遺伝子によりコードされる事実[2)8)]と合致す

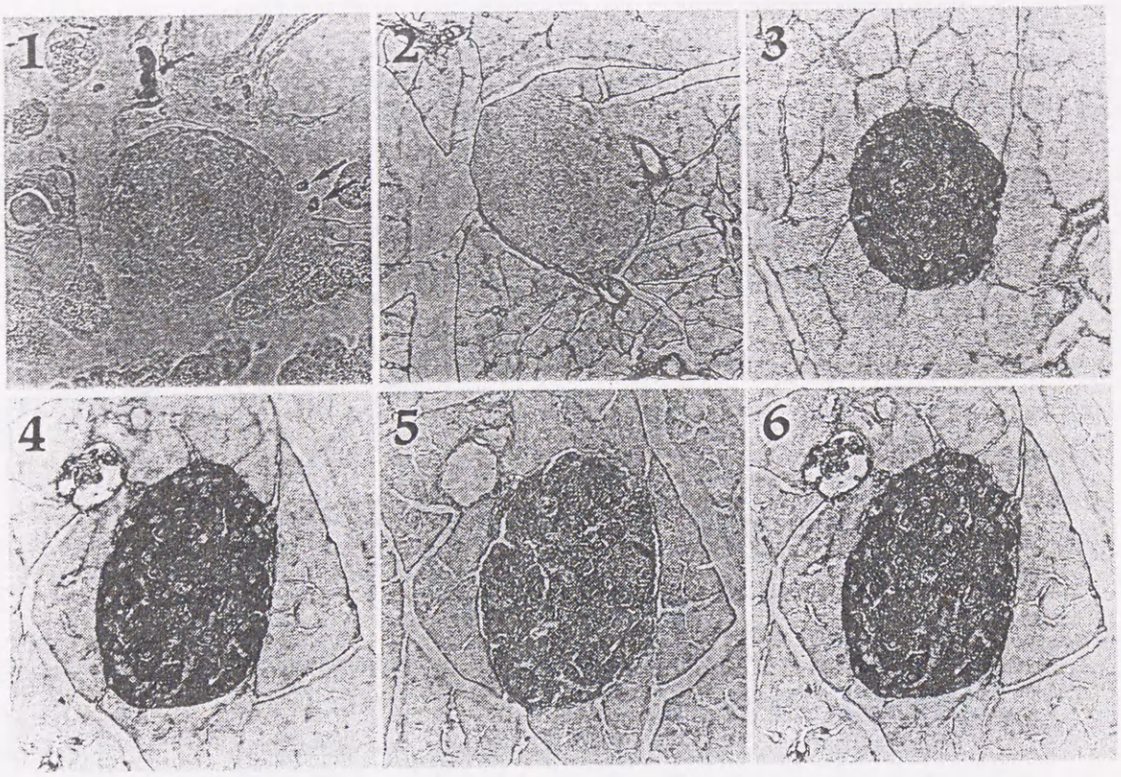


Fig. 3 Immunohistochemical detection of VIP, PHM-27 and insulin in mouse pancreas. Sections of the control (1, 2, 3) and transgenic (4, 5, 6) pancreas were incubated with the antiserum against VIP (1, 4), PHM-27 (2, 5), or insulin (3, 6) followed by visualization with the avidin-biotin peroxidase method. VIP-positive nerve fibres and ganglions close to the periphery of nontransgenic islets are indicated by arrows (1).

る。PHM-27 と VIP はその構造および作用が類似しており，最近では共通のリセプターを介して生理作用を発揮する可能性も考えられている。インスリンの免疫組織染色では両者に差を認めなかった（Fig. 3-3, 6）。

VIP, PHM-27 前駆体はシグナルペプチドを持つことから，このトランスジェニックマウスにおいて産生された VIP, PHM-27 はインスリンと同様に膵ランゲルハンス島細胞より分泌されることが予想される。そこで次にトランスジェニックマウスおよびコントロールマウスより膵ランゲルハンス島を分離培養し，培地中に放出された VIP をラジオイムノアッセイ(RIA)により測定した。すると Fig. 4 のように，低濃度 (2.7mM) グルコース存在下ではトランスジェニックマウスの膵ランゲルハンス島 100 個当たり 1 時間で 12.5±6.9 femtomol の VIP が培地中に分泌された。高濃度 (16.7mM) グルコース存在下ではそれよりずっと多い，100.2±26.9 femtomol の VIP が培地中に分泌された。このようにトランスジェニックマウスの膵ランゲルハンス島からの VIP 分泌は，高濃度グルコース刺激によって促進されることが明らかになった。このように分泌された VIP は *in vivo* でオートクライン機構により膵 β 細胞自身に作用すると考えられる。それに対し，コントロールマウスの膵ランゲルハンス島からの VIP 分泌はいずれの点においても検出限界以下であった。

3. 膵 β 細胞において産生・分泌された VIP および PHM-27 はグルコース誘起性インスリン分泌反応を速やかに増強し耐糖能を改善する

トランスジェニックマウスの膵ランゲルハンス島において産生された VIP と PHM-27 が全身の血糖調節に及ぼす作用について明らかにするため

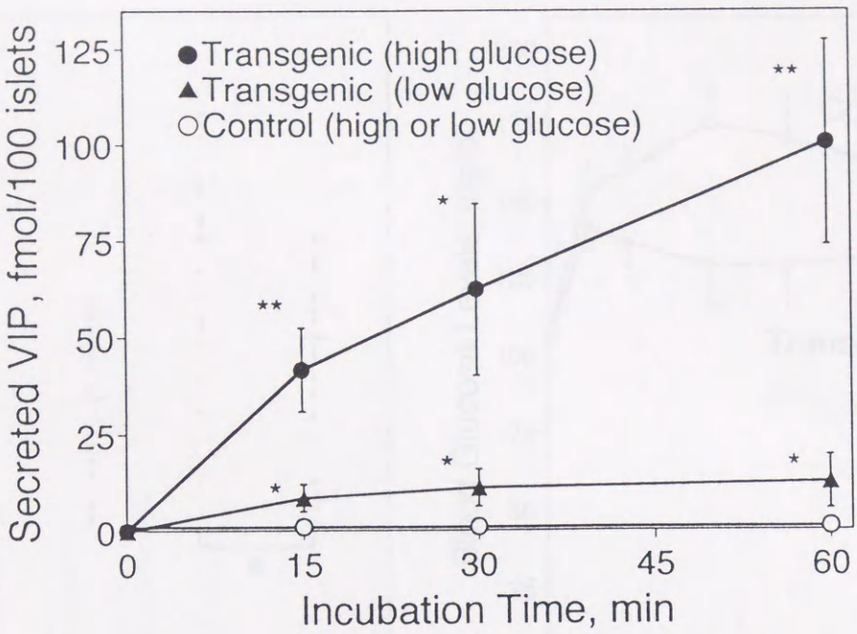


Fig. 4 VIP secretion from isolated islets under low or high glucose concentrations. 100 islets of each type were exposed to media containing low glucose (2.7mM glucose) or high glucose (16.7mM glucose), and then medium samples were removed at the indicated time and assayed for radioimmunoassay of VIP. N=3 for each point. *P<0.05, **P<0.01 (different from control). Vertical bars indicate standard errors of the mean (SEM).

空腹時および随意摂食時の血糖値を測定した(Fig. 5)。すると空腹時の血糖値はコントロールマウスとトランスジェニックマウスで有意差を認めないものの，随意摂食時においては，コントロールマウスが155±6mg/dlと高いのに比較し，トランスジェニックマウスにおいては128±4mg/dlと血糖値の有意な低下を認めた。これより，トランスジェニックマウスにおいて血糖値上昇に対する抵抗性が存在することが考えられた。

この点をさらに詳しく検討するため，グルコースをマウス腹腔内に注射し，糖負荷試験を行った(Fig. 6)。トランスジェニックマウスの血糖値は15分値をピークとしてその後直ちに下降し始め，30分値から120分値の間でコントロールマウスに比べ有意に低値を示した。

この糖負荷試験の結果からわれわれはトランスジェニックマウスにおいてインスリン分泌が高まっている可能性を考え，糖負荷試験の各点においてマウスをサクリファイスして血清を採取しインスリンをRIAにより定量した(Fig. 7)。すると0分時の血清インリスン値は差を認めないが，15分値においてはコントロールマウスの0.76±0.1ng/mlに比べ，トランスジェニックマウスではその約2.8倍の2.13±0.52ng/mlという有意に高いインスリン値を示した。以上からトランスジェニックマウスの膵ランゲルハンス島において産生されるVIPおよびPHM-27がグルコース刺激に対応したインスリン分泌反応を速やかに増強し耐糖能を改善することが明らかになった。

次にコントロールマウスおよびトランスジェニックマウスの両者に対して同じように70%膵切除を行い，1週間後に糖負荷試験を行った(Fig. 8)。するとコントロールマウスの血糖値は3時間後に165±15mg/dlとなお高い値を示したのに対して，トランスジェニックマウスでは112±18mg/dlとほぼ糖負荷前の値にまで下降していた。90分値以降で両者に有意差を認め，70%膵切除による耐糖能低下はトランスジェニックマウスにお

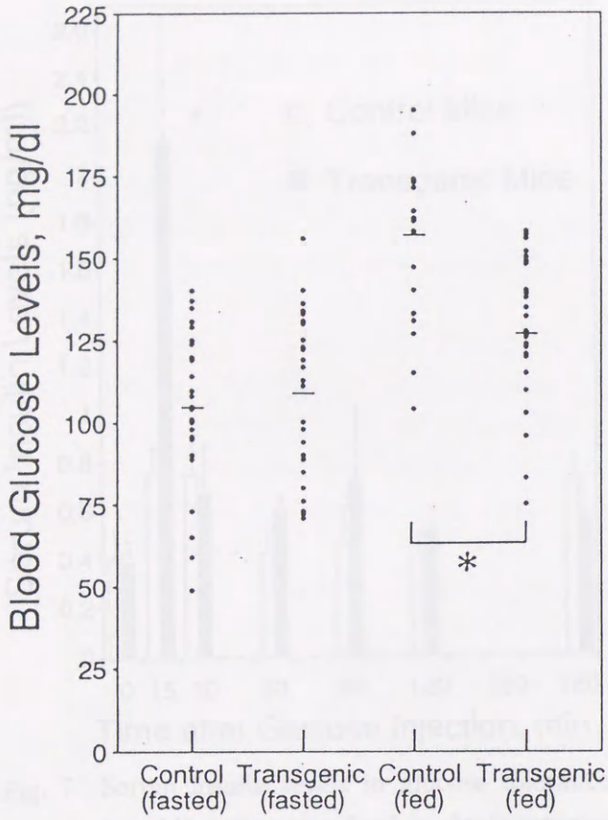


Fig. 5 Blood glucose analyses. The mean±SEM in each column was; 106±4 mg/dl (control, fasted, n=29), 110±5 mg/dl (transgenic, fasted, n=29), 155±6 mg/dl (control, fed, n=20), 128±4 mg/dl (transgenic, fed, n=29). *P<0.001 (different from control).

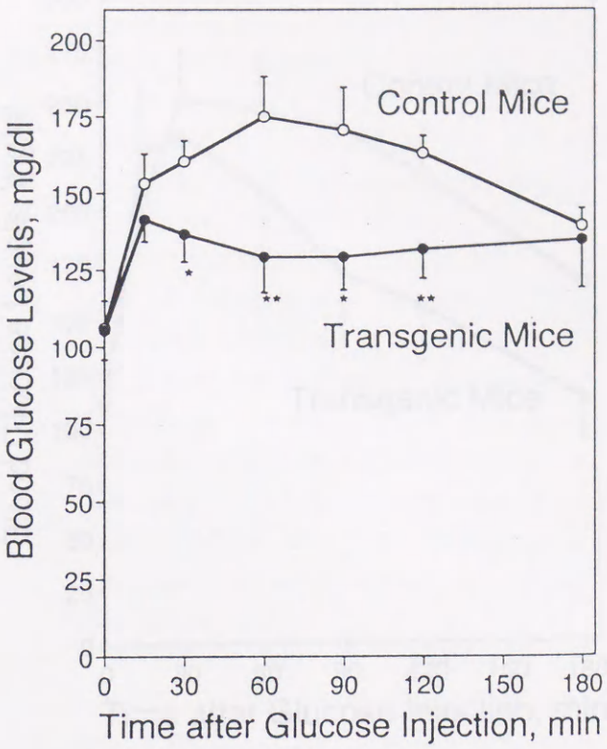


Fig. 6 Glucose tolerance test. Transgenic mice (Transgenic) and their nontransgenic siblings (Control) were administered an intraperitoneal injection of 2 mg of glucose per g (body weight). N=8 (transgenic) and 8 (control). *P<0.05, **P<0.02 (different from control). Vertical bars indicate SEM.

いて軽減されていると考えられた。

考　察

インスリンプロモーターを用いたトランスジェニックマウス作製の報告はこれまでに20数例を数えるが，その多くの例で導入遺伝子産物により糖尿病が引き起こされている[9~11]。このヒト VIP 遺伝子導入トランスジェニックマウスのようにインスリン分泌を促進し耐糖能を改善するようなトランスジェニックマウス作製の報告は Epstein らの酵母ヘキソキナーゼ遺伝子導入マウス作製の報告[12]に次いで2例め，分泌蛋白をコードする遺伝子としては最初の報告である。

VIP は膵ランゲルハンス島近傍の神経線維や神経節内に密に存在すること（Fig. 3-1 および文献5），ブタにおいて迷走神経に電気的刺激を与えると膵内神経終末より VIP が放出されること[13]などから，*in vivo* で膵 β 細胞からのインスリン分泌を促進する可能性が示唆されてきた。このトランスジェニックマウスでの知見は VIP をニューロトランスミッターとする，インスリン分泌の神経性調節が生体内で実際に機能し得ることを示唆する。また VIP によるインスリン分泌の促進は，食事後それほど血糖が上昇していない段階でインスリン分泌が起こるという現象[14]をも説明し得る。

本トランスジェニックマウスにおいては，ヘキソキナーゼ遺伝子導入マウス[12]の場合と異なり，導入遺伝子の血糖降下作用は血糖値が低い領域では認められなかった（Fig. 5, 6）。これは（1）低濃

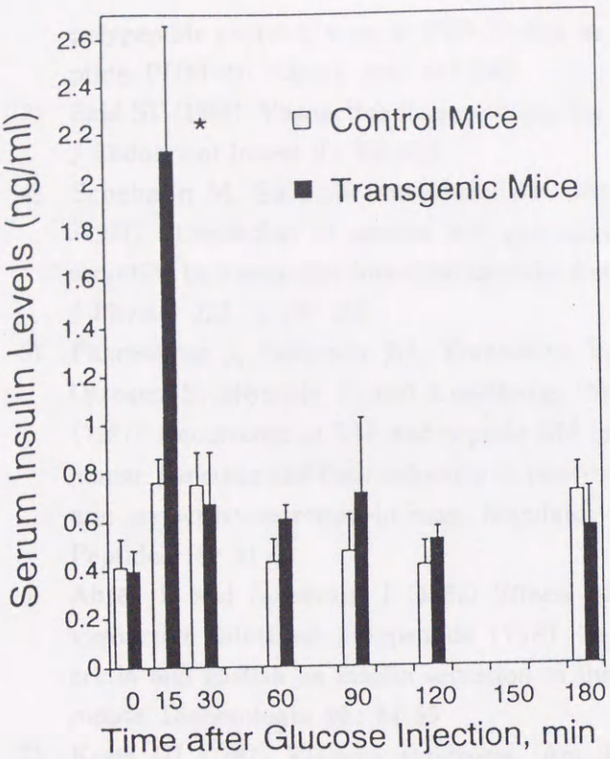


Fig. 7 Serum insulin levels in glucose tolerance test. Mice were sacrificed to obtain samples for measurement of serum insulin levels at each point after glucose administration. For each point, at least six determinations were obtained. *P<0.02 (different from control). Vertical bars indicate SEM.

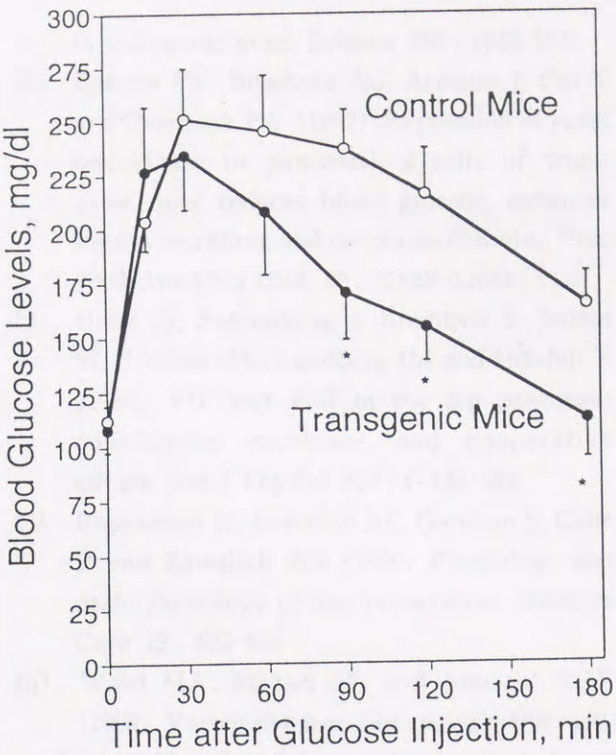


Fig. 8 Glucose tolerance test in the 70% depancreatized mice. N=7 (transgenic) and 7 (control). *P<0.05 (different from control). Vertical bars indicate SEM.

度グルコース存在下ではトランスジェニックマウスの膵ランゲルハンス島からのVIP分泌量が非常に低いこと(Fig. 4), (2) 低濃度グルコース存在下におけるVIPのインスリン分泌増強作用は非常に小さいこと[15]などにより説明され得る。

MODY (Maturity-onset diabetes of the young) 症候群ではグルコキナーゼ遺伝子の変異[16]によりインスリン分泌が低下していることが見出されている。またインスリン非依存型糖尿病 (NIDDM: Non-insulin-dependent diabetes mellitus) においては, グルコース刺激によるインスリン分泌が低下していることが指摘されている[17]。そのようなインスリン分泌障害型の糖尿病に対してVIPを将来的に糖尿病治療の薬剤として使用し得る可能性もある。VIP投与により低血糖を招来する可能性は少ないこと(Fig. 5, 6), さらにVIPがスルホニルウレア剤などと異なり, 天然のインスリン分泌促進性物質であることも有利な点である。将来, 効率的な膵β細胞への遺伝子導入が可能になれば, ヒトVIP遺伝子を用いた糖尿病遺伝子治療の可能性も考えられる。本トランスジェニックマウスにおいて少なくとも生後1年まではVIP遺伝子導入による副作用は一切認められない。しかし, 副作用発現にさらに長期間を要する可能性もあり, その検討は必要であろう。なお, 本論文ではライン330について解析を行ったが, 最近になりライン361および366についても糖負荷試験と血清インスリン値測定を行い, ほぼ同様の解析結果が得られたので参照されたい[18]。

文 献

1) Said SI and Mutt V (1970) Polypeptide with broad biological activity: Isolation from small intestine. Science 169: 1217-1218

2) Itoh N, Obata K, Yanaihara N and Okamoto H (1983) Human preprovasoactive intestinal

polypeptide contains a novel PHI-27-like peptide, PHM-27. Nature 304 : 547-549

3) Said SI (1986) Vasoactive intestinal peptide. J Endocrinol Invest 9 : 191-200

4) Schebalin M, Said SI and Makhlouf GM (1977) Stimulation of insulin and glucagon secretion by vasoactive intestinal peptide. Am J Physiol 232 : E 197-200

5) Fahrenkrug J, Pederson JH, Yamashita Y, Ottesen B, Hökfelt T and Lundberg JM (1987) Occurrence of VIP and peptide HM in human pancreas and their influence on pancreatic endocrine secretion in man. Regulatory Peptides 18 : 51-61

6) Ahrén B and Lundquist I (1981) Effects of vasoactive intestinal polypeptide (VIP), secretin and gastrin on insulin secretion in the mouse. Diabetologia 20 : 54-59

7) Krejs GJ (1987) VIPoma syndrome. Am J Med 82 : 37-47

8) Yamagami T, Ohsawa K, Nishizawa M, Inoue C, Gotoh E, Yanaihara N, Yamamoto H and Okamoto H (1988) Complete nucleotide sequence of human vasoactive intestinal peptide/PHM-27 gene and its inducible promoter. Ann NY Acad Sci 527 : 87-102

9) Sarvetnick N, Liggitt D, Pitts SL, Hansen SE and Stewart TA (1988) Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma. Cell 52 : 773-782

10) Epstein PN, Overbeek PA and Means AR (1989) Calmodulin - induced early - onset diabetes in transgenic mice. Cell 58 : 1067-1073

11) Stewart TA, Hultgren B, Huang X, Pitts-Meek S, Hully J and MacLachlan NJ (1993) Induction of type I diabetes by interferon-α in transgenic mice. Science 260 : 1942-1946

12) Epstein PN, Boschero AC, Atwater I, Cai X and Overbeek PA (1992) Expression of yeast hexokinase in pancreatic β cells of transgenic mice reduces blood glucose, enhances insulin secretion, and decreases diabetes. Proc Natl Acad Sci USA 89 : 12038-12042

13) Holst JJ, Fahrenkrug J, Knuhtsen S, Jensen SL, Nielsen OV, Lundberg JM and Hökfelt T (1987) VIP and PHI in the pig pancreas : coexistence, corelease, and cooperative effects. Am J Physiol 252 : G 182-189

14) Rasmussen H, Zawalich KC, Ganesan S, Calle R and Zawalich WS (1990) Physiology and pathophysiology of insulin secretion. Diabetes Care 13 : 655-666

15) Wahl MA, Straub SG and Ammon HPT (1993) Vasoactive intestinal polypeptide-augmented insulin release : actions on ionic fluxes and electrical activity of mouse islets. Diabetologia 36 : 920-925

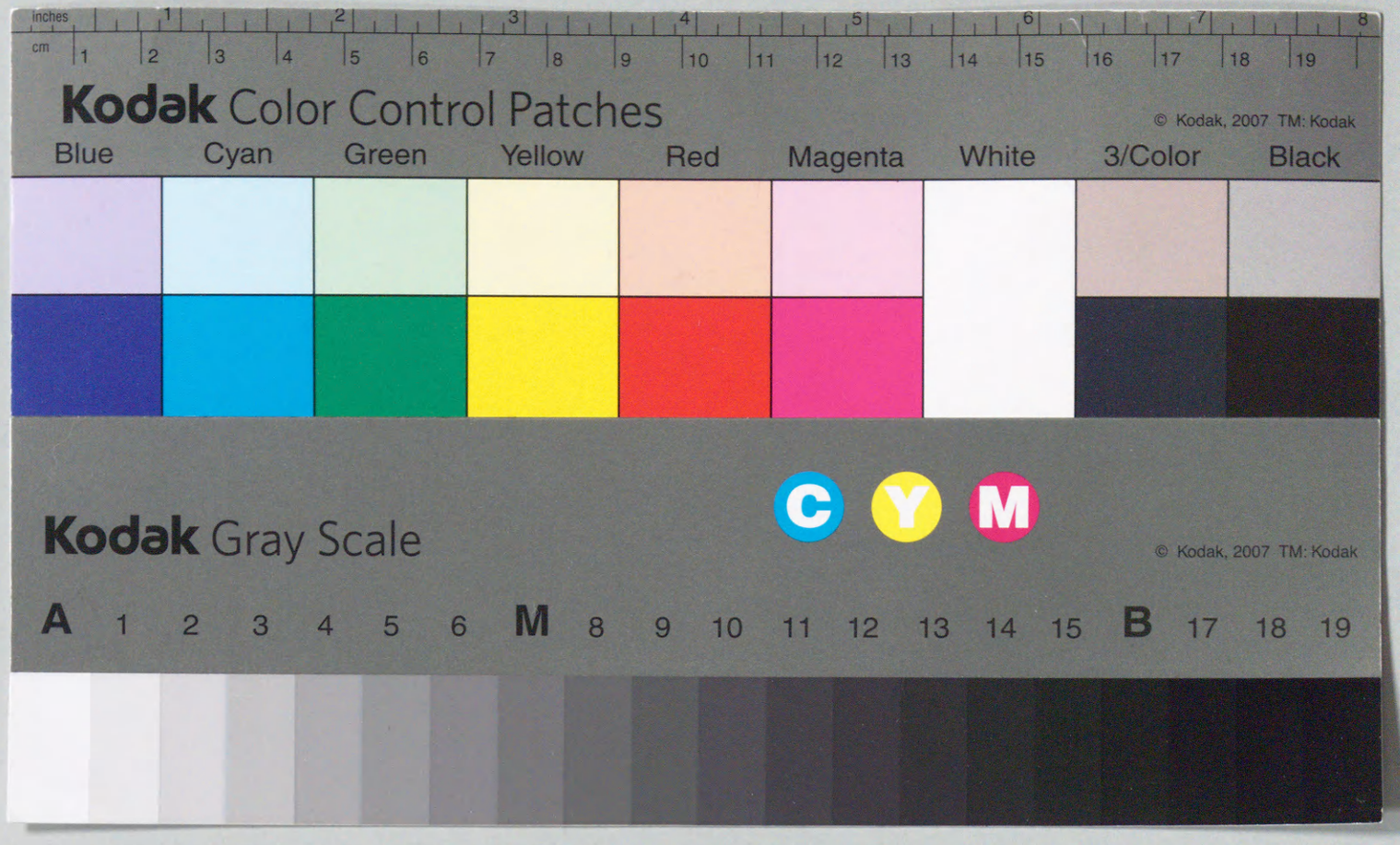
16) Vionnet N, Stoffel M, Takeda J, Yasuda K, Bell GI, Zouali H, Lesage S, Velho G, Iris F, Passa Ph, Froguel Ph and Cohen D (1992) Nonsense mutation in the glucokinase gene causes early-onset type diabetes of young people. Nature 356 : 721-722

17) Pfeifer MA, Halter, JB and Porte D (1981) Insulin secretion in diabetes mellitus. Am J Med 70 : 579-588

18) Kato I, Suzuki Y, Akabane A, Yonekura H, Tanaka O, Kondo H, Takasawa S, Yoshimoto T and Okamoto H (1994) Transgenic mice overexpressing human vasoactive intestinal peptide gene in pancreatic β cells : evidence for improved glucose tolerance and enhanced insulin secretion by VIP and PHM-27 *in vivo*. J Biol Chem *in press*.

謝　辞

稿を終えるにあたり、終始御指導と御鞭撻を賜りました東北大学医学部脳神経外科学講座　吉本高志教授に厚く御礼申し上げます。また、終始御指導、御教示頂きました東北大学医学部医化学第一講座　岡本　宏教授、米倉秀人助教授、高澤　伸講師、加藤一郎助手に深く感謝の意を表します。併せて、本研究を遂行するにあたり御協力頂きました東北大学医学部医化学第一講座の諸兄に厚く御礼申し上げます。


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19